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CATALYSIS OF TYROSINE OXIDATION AND IODINATION BY HORSERADISH PEROXIDASE

by

© ISOBEL MARGARET RALSTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

The pH dependencies of the oxidations of L-tyrosine and 3,5-diiodo-L-tyrosine by both compounds I and II of horseradish peroxidase were investigated at 25°C and ionic strength 0.11 by a stopped-flow procedure. The ionization of an enzyme acid group of $pK_a \sim 5$ has a pronounced effect on the kinetics of compound I reactions; the deprotonated form of this group is most reactive. For the compound II reactions, an enzyme dissociation at pH 8.6 exerts the greatest influence on the rate. A second compound II acid group of lower pK_a also has a slight effect on the kinetics of substrate oxidation. Protonation of the phenolic group of the substrate is favoured for the reactions of both compounds I and II; it was observed that the total substrate charge plays an important role. A stoichiometric investigation showed that either tyrosine or diiodotyrosine is required in only a 1 M equivalent for the two-electron reduction of compound I to ferric enzyme.

The horseradish peroxidase-catalyzed reaction of iodide with tyrosine was studied as a function of pH. It is proposed that a mechanism involving molecular iodine oxidation to an "$I^{\oplus}$" species and its subsequent reaction with tyrosine is operative at low pH. The change in kinetics at pH ~ 5 is attributed to deprotonation of a compound I acid group, accompanied by a rapid decrease in the rate of iodide

oxidation and an increase in tyrosine oxidation. It was noted that the enzyme does not catalyze efficiently the iodination of tyrosine under basic conditions; tyrosyl radicals likely recombine to yield bityrosine before the oxidation of iodide takes place.

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CHAPTER I.

INTRODUCTION

1.1 Forms of Iron in Biological Systems

It is not surprising that the element iron, because of its abundance on our planet, has been incorporated into many physiological systems. Many different biological molecules which utilize this metal, each with a specific function, have evolved. These may be divided into five major categories as shown in Table 1.1 (a) iron storage forms; (b) iron transport forms; (c) oxygen carriers; (d) enzymes, and (e) electron carriers.

In the more complex classes of animal life iron must be stored and readily available for the biosynthesis of oxygen and electron carriers. Ferritin (1a-4) and hemosiderin (1a,4) sequester iron (III) into non-toxic and soluble forms. When required, the transport of iron then takes place via blood serum transferrin, also known as siderophilin or β_1 metal-binding globulin (1b,5-7). In certain fungi and antinomycetes, iron conveyance occurs by means of various trihydroxamic acids (8,9); whereas, enterobactins (enterochelins) are responsible for microbial transfer and metabolism of iron (8,10,11).

Hemoglobin and myoglobin are the oxygen carriers in red blood and muscle cells respectively. Both are found in vertebrates; however, some crustaceans and roots of leguminous plants (i.e. leghemoglobin) also contain hemoglobin. In

Table 1.1 Forms of Iron in Biological Systems

Iron Storage	Iron Transport	Oxygen Carriers	Enzymes	Electron Carriers
Ferritin	Transferrins	Hemoglobin	Heme Enzymes	Cytochromes
Hemosiderin	Trihydroxamic Acids	Myoglobin	Non-heme Enzymes	Iron-Sulphur Electron Carriers
	Enterobactins	Hemerythrin	Iron-sulphur Enzymes	

either case, the iron is bound to a porphyrin as illustrated in Fig. 1.1. For hemoglobin and myoglobin this structure is known as heme or ferroprotoporphyrin IX; whereas, other proteins may contain hematin or hemin, the ferriprotoporphyrin IX equivalent. Generally speaking, the term heme may refer to any valent form of iron bound to protoporphyrin. Oxygen transport in a few simple invertebrates proceeds via the non-heme iron protein, hemerythrin (12,13).

There are many iron-containing enzymes which do not have iron-sulphur clusters. These may be subdivided into two groups, the heme and the non-heme enzymes. The list of heme enzymes includes the following: peroxidase, catalase, tryptophan and indoleamine dioxygenases, sulphite and cytochrome c oxidases, lactate dehydrogenase and cytochrome P-450. The electron-carrying cytochromes also contain heme. Lipoxygenase and most dioxygenases (14), with the exception of the two already mentioned, are non-sulphur, non-heme, iron-containing enzymes. In addition, a special group of enzymes and proteins contain both iron and sulphur (15). Nitrogenase, NADH, succinate and dihydroorotate dehydrogenases, xanthine and aldehyde oxidases, biquinone - cytochrome c and ferredoxin - nitrite reductases are the major iron-sulphur enzymes. Rubredoxins, ferredoxins and "high-potential" iron-sulphur proteins all act as electron carriers for various physiological processes.

Having established the significance of iron in biological systems, the author's attention will focus on one of

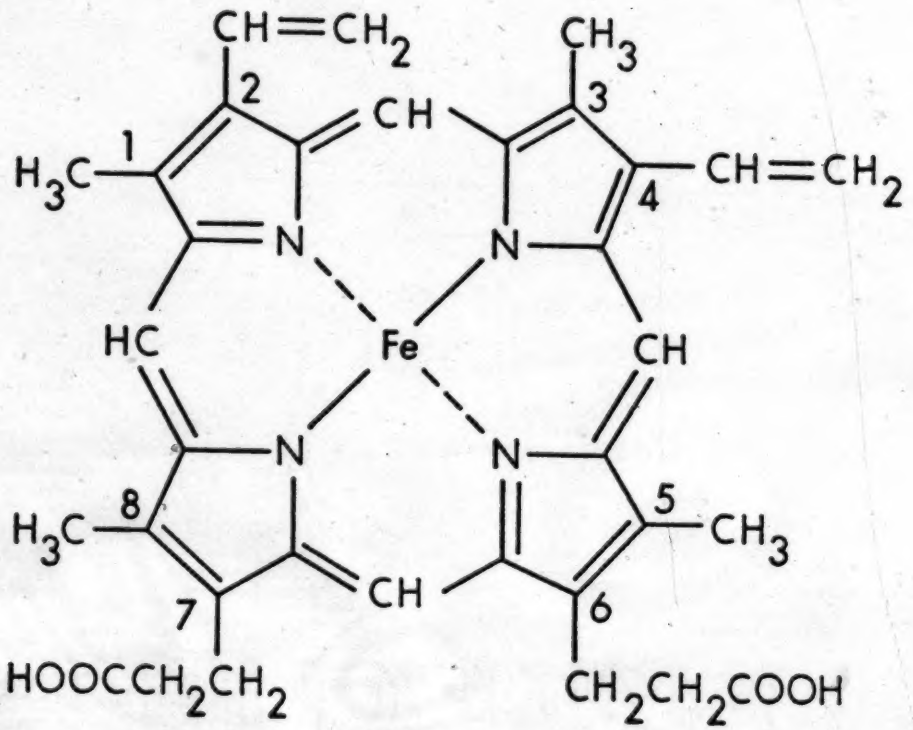


Fig. 1.1 Structure of iron protoporphyrin IX.

the heme-containing enzymes, peroxidase, and in particular on several reactions either catalyzed by horseradish peroxidase or involving its intermediates. For comparative purposes, the Appendix is entirely devoted to a literature survey of another heme enzyme, L-tryptophan-2,3-dioxygenase, whose properties, functions and catalytic mechanism of action significantly differ from those of horseradish peroxidase.

1.2 Peroxidases

Peroxidases (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) are classified as the group of enzymes which catalyze the oxidation of numerous organic and inorganic compounds by hydrogen peroxide or ROOH. Many peroxidases with diverse physiological functions are found in nature. They exist in most plants (16); worthy of note is the root of the horseradish plant in which the enzyme is highly concentrated. The function(s) of these plant enzymes is not yet clearly understood, although investigations have shown that indoleacetic acid, a plant growth hormone, reacts quickly with turnip and horseradish peroxidases (17). Many animal tissues and fluids also contain peroxidases. Among the better studied are thyroid peroxidase which takes part in the production of thyroid gland hormones, myeloperoxidase of the leucocytes and lactoperoxidase which was first isolated from bovine milk. Other peroxidases are present in molds and yeasts; chloroperoxidase is found in *Caldariomyces fumago* and baker's yeast is a source of cytochrome c peroxidase.

The prosthetic group of most peroxidases is ferriprotoporphyrin IX (Fig. 1.1). A notable exception is glutathione peroxidase for which selenium is essential for activity (18). In spite of the similarities among the peroxidases, it should be remembered that the enzymes from various sources often have very different properties. Horseradish peroxidase has become one of the more intensively studied peroxidases because of its ready availability and its purity and stability upon isolation.

1.3 Horseradish Peroxidase

1.3.1 Properties

There are seven major and thirteen minor isoenzymes of horseradish peroxidase (20). Discussion will be limited to the isoenzyme of isoelectric point ~9 (isoenzyme C or III b) because of its relative abundance in horseradish roots (19, 20).

Horseradish peroxidase is a glycoprotein of molecular weight 44,000 (20), a carbohydrate content of 18% by weight (21), and 43% α-helical structure (22). One mole of ferriprotoporphyrin IX is contained per mole of enzyme. The presence of Ca^{2+} ions has been detected; presumably these ions help to maintain the protein structure near the heme environment (23,24). The amino acid sequence has been determined, yielding valuable information about its structure (20,25). A total of 308 amino acid residues were found in a single polypeptide chain, along with eight neutral carbohydrate

side chains attached to asparagine residues. Four disulphide and three histidyl residues are present; one of the histidines occupies the fifth coordination position of the ferric ion (26-30).

The controversy over the presence or absence of water in the sixth coordination position of the horseradish peroxidase ferric ion has not yet been resolved. Nmr results have yielded contradictory evidence (31,32). The physical changes accompanying the alkaline transition of horseradish peroxidase (pK ~ 11) were originally attributed to the conversion of water in the sixth position to hydroxide ion (33,34); however, an alternative explanation has been offered. It was suggested that a distal histidine coordinates to the ferric ion upon ionization at pH ~ 11 (35).

1.3.2 Intermediates of the Enzymatic Cycles

Two intermediates of horseradish peroxidase, commonly called compound I and compound II, are encountered during the enzymatic oxidation of substrates by hydrogen peroxide. Compound I is produced by the addition of a stoichiometric amount of hydrogen peroxide to the "native", ferric enzyme; both oxidizing equivalents of the hydrogen peroxide are acquired by the enzyme during this step. A subsequent one-electron reduction of compound I by a suitable reducing agent produces compound II; its state of oxidation is intermediate to those of compound I and ferric enzyme. The three species of horseradish peroxidase are easily distinguished by their Soret and visible spectra, as shown in Fig. 1.2.

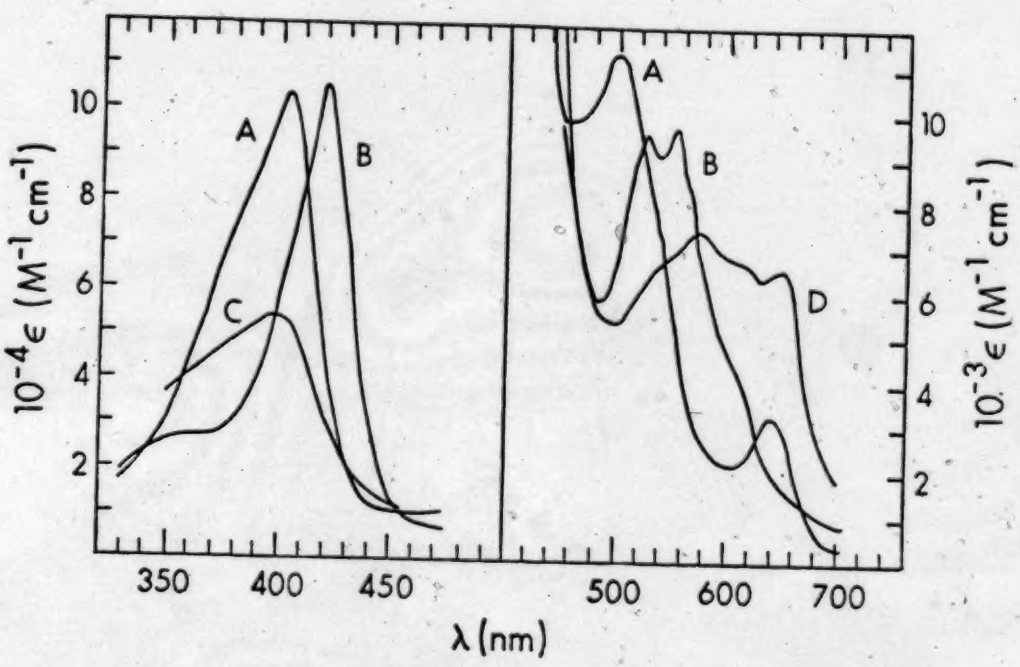


Fig. 1.2 A, Soret and visible spectra of ferric horseradish peroxidase (36); B, Soret and visible spectra of horseradish peroxidase compound II (36); C, Soret spectrum of horseradish peroxidase compound I (37); D, Visible spectrum of horseradish peroxidase compound I (38).

The major transitions observed between 390 and 430 nm (the Soret bands), and the distinct isosbestic points facilitate the study of each intermediate.

The enzymatic cycle for horseradish peroxidase is dependent on the nature of the reducing substrate. There are three distinct cycles which account for the stoichiometries of the reactions of the horseradish peroxidase intermediates with three different groups of substrates. The following mechanism describes the series of reactions during the horseradish peroxidase-catalyzed oxidation of ferrocyanide (39), *p*-aminobenzoic acid (40,41) or nitrite ion (42):

$$[1.1] \quad HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O$$

$$[1.2] \quad HRP\text{-}I + S \longrightarrow HRP\text{-}II + S_{ox} \qquad \text{I}$$

$$[1.3] \quad HRP\text{-}II + S \longrightarrow HRP + S_{ox}$$

where HRP, HRP-I, HRP-II, S and S_{ox} represent ferric horseradish peroxidase, compound I, compound II, substrate and oxidized substrate respectively.

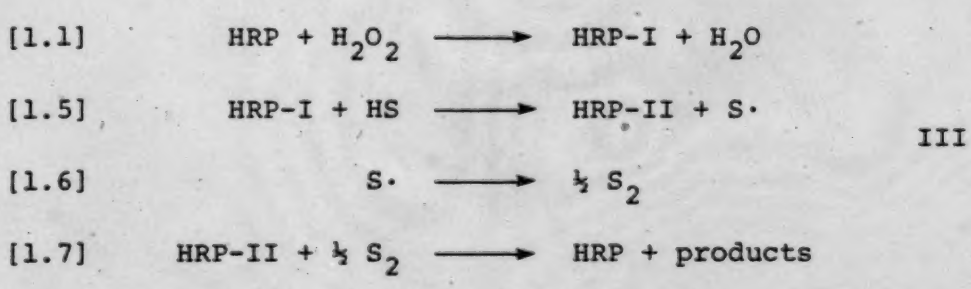
For the oxidation of iodide (37) and bisulphite (42) ions, the reaction sequence proceeds without detectable formation of compound II:

$$[1.1] \quad HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O$$

$$[1.4] \quad HRP\text{-}I + S \longrightarrow HRP + S_{ox,ox} \qquad \text{II}$$

Reaction 1.4 is a two-electron redox reaction and $S_{ox,ox}$ contains both oxidizing equivalents. Compound II, by itself, also reacts with these substrates (42,43); however, under normal conditions, the reaction proceeds as shown in scheme II.

The third type of enzymatic cycle accounts for the oxidation of *p*-cresol (44). When stoichiometric amounts of substrate, HS, are added, the following steps may explain the fact that only a 1 M equivalent of substrate is required for the two-electron reduction of compound I to native enzyme:



[1.1] $HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O$

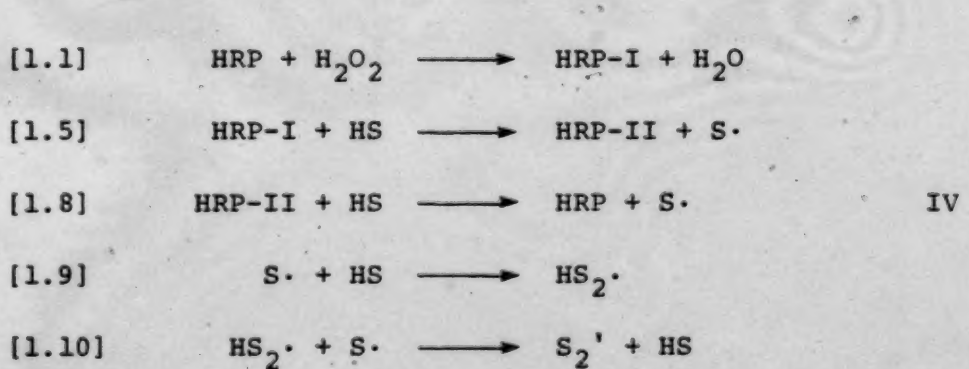
[1.5] $HRP\text{-}I + HS \longrightarrow HRP\text{-}II + S\cdot$

[1.6] $S\cdot \longrightarrow \frac{1}{2} S_2$ III

[1.7] $HRP\text{-}II + \frac{1}{2} S_2 \longrightarrow HRP + products$

where S_2 is a 2,2'-dihydroxy-5,5'-dimethylbiphenyl (44).

If the *p*-cresol and hydrogen peroxide are in excess, the enzyme recycles until either substrate is depleted, as shown by:



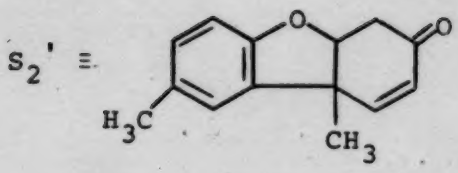
[1.1] $HRP + H_2O_2 \longrightarrow HRP\text{-}I + H_2O$

[1.5] $HRP\text{-}I + HS \longrightarrow HRP\text{-}II + S\cdot$

[1.8] $HRP\text{-}II + HS \longrightarrow HRP + S\cdot$ IV

[1.9] $S\cdot + HS \longrightarrow HS_2\cdot$

[1.10] $HS_2\cdot + S\cdot \longrightarrow S_2' + HS$



S_2' is Pummerer's ketone which cannot be oxidized further. The reaction sequence may be complicated by the oxidation of $HS_2\cdot$ by either compound I or compound II to yield S_2' (44).

Besides the three species of horseradish peroxidase which actively take part in the enzymatic cycles, two other redox forms have been studied. The reduction of the ferric enzyme by sodium dithionite yields ferroperoxidase. Compound III (analogous to either oxygen-ferroperoxidase or superoxide-ferriperoxidase) may be produced by the reactions of either compound II with hydrogen peroxide or ferroperoxidase with oxygen. In order of decreasing oxidation state, the five redox species of horseradish peroxidase are: compound III, compound I, compound II, ferriperoxidase and ferroperoxidase.

1.3.3 Compound I and its Reactions

The structure and mechanism of formation of compound I have not yet been clearly defined. The available experimental data indicate that the iron is oxidized to ferryl ion, Fe(IV) (45), and that an oxygen atom occupies the sixth coordination position (46,47). The major disagreement over the nature of compound I, however, concerns the location of

the second oxidizing equivalent. It has been proposed that either a porphyrin π-cation radical or a free radical on an amino acid residue is formed. A strong radical epr signal for compound I of cytochrome c peroxidase is indicative of the protein radical concept (48); but for horseradish peroxidase compound I, only a weak epr signal was detected (49). An experiment with cobaltous octaethylporphyrin has shown that a stable π-cation radical, with an optical spectrum similar to compound I of horseradish peroxidase, is formed upon a two-electron oxidation (50). Several other investigations using epr (45,51-53) and nmr (54-56) techniques have been conducted in an attempt to resolve the problem. Unfortunately, none is conclusive and neither the π-cation nor the protein free radical concept may be eliminated at the present time.

An acid group of horseradish peroxidase (perhaps a distal aspartate residue (57)) plays a significant role in the formation of compound I from ferriperoxidase and hydrogen peroxide (58). The possibility of hemin propionate side chain participation was ruled out when peroxidase, reconstituted with hemin containing esterified propionate groups, still produced compound I (57). A high-pressure stopped-flow study is suggestive of the involvement of a negatively-charged basic group (aspartate) in the reaction of ferric enzyme with hydrogen peroxide (59).

The reactions of various substrates with compound I have been studied as a function of pH, to gain some insight

into the acid group(s) of compound I which participates in the oxidation of substrates. Fig. 1.3 demonstrates the pH dependencies of the second order rate constant, k_{app}, for the pseudo-first order reactions of compound I with the given substrates. One common feature is the enzymatic $pK_a \sim 5$ which accounts for the acid catalysis of iodide (37), nitrite (42), bisulphite (42), *p*-aminobenzoic acid (40) and ferrocyanide (39,58) and the base catalysis of *p*-cresol (60). The significance of these results will be elaborated upon in Chapter II.

1.3.4 Compound II and its Reactions

The one-electron reduction of compound I yields compound II, which also has a ferryl type structure (61). The reactions of compound II are generally slower than those of compound I as shown in Fig. 1.4. The oxidations of *p*-aminobenzoic acid (40), ferrocyanide (39,58) and *p*-cresol (62) are catalyzed by protonation of an enzyme group of $pK_a \sim 8.6$ whereas, the iodide (43), nitrite (42) and bisulphite (42) oxidations are affected by an enzymatic pK_a outside of the pH range of study. The relevance of these studies will be discussed in Chapter III.

The two most recent review articles on peroxidases (63,64), include interesting sections on horseradish peroxidase.

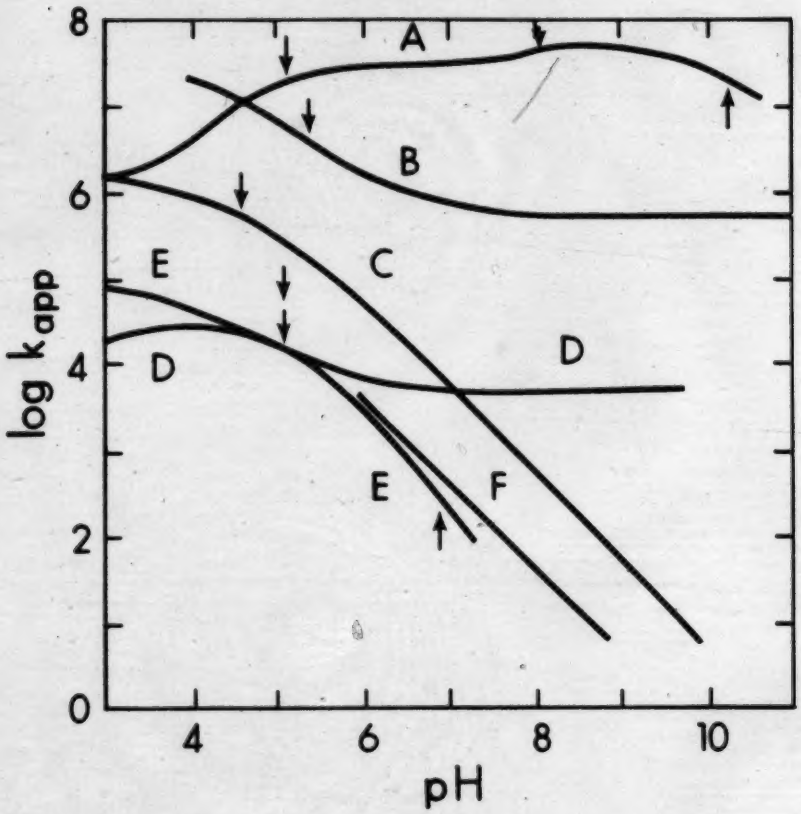


Fig. 1.3 Log (k_{app}) versus pH profiles for reactions of horseradish peroxidase compound I. Substrates: A, *p*-cresol (60); B, ferrocyanide ion (39,58); C, iodide ion (37); D, *p*-aminobenzoic acid (40); E, bisulphite ion (42); F, nitrous acid (42). The compound I pK_a values are represented by ↓. The substrate pK_a values are indicated by ↑. Units of k_{app} are $M^{-1}s^{-1}$.

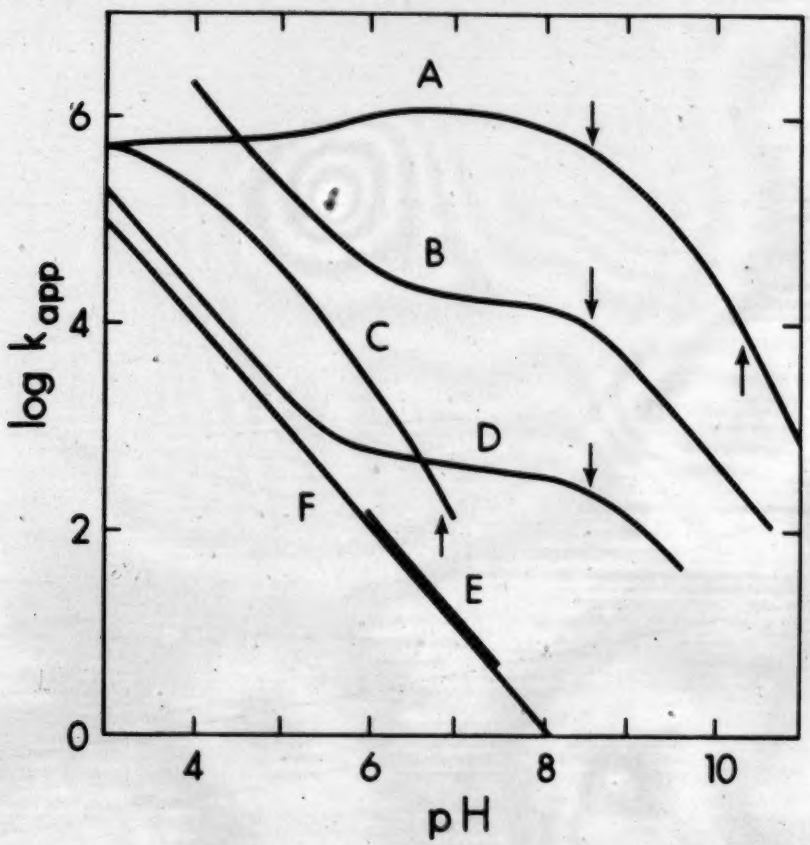


Fig. 1.4 Log (k_{app}) versus pH profiles for reactions of horseradish peroxidase compound II. Substrates: A, *p*-cresol (62); B, ferrocyanide ion (39,58); C, bisulphite ion (42); D, *p*-aminobenzoic acid (40); E, nitrous acid (42); F, iodide ion (43). The compound II pK_a values are represented by ↓. The substrate pK_a values are indicated by ↑. Units of k_{app} are $M^{-1}s^{-1}$.

1.4 Peroxidase-Catalyzed Iodination of Tyrosine

The reaction of the amino acid, L-(-)-tyrosine with iodide is an essential step in the production of the thyroid gland hormones, thyroxin and 3,3',5-triiodothyronine, as shown in Fig. 1.5. Physiologically, the iodination does not involve free tyrosine; instead, the tyrosyl residues of a large, soluble protein, thyroglobulin, react with free iodide to produce monoiodotyrosine and diiodotyrosine (65). Still within the thyroglobulin structure, the iodotyrosines couple to form the hormones (66). Afterwards these hormones are released into the bloodstream as free amino acids by thyroglobulin proteolysis (66).

In the biological system, the conversion of tyrosine to the hormones is catalyzed by thyroid peroxidase. The enzyme is required for both the tyrosine iodination and the coupling reaction (65,66). The essential hydrogen peroxide for these steps is perhaps generated from a system containing NADPH-cytochrome c reductase, vitamin K_3 and oxygen; however, this hypothesis has not yet been proven (66).

Although most thyroid diseases are the indirect result of an improperly functioning pituitary gland, there are several rare types of hypothyroidism which are caused by defects in the sequence of reactions leading to hormone biosynthesis; congential goitrous cretinism has been linked to the absence of thyroid peroxidase and Pendred's syndrome may be due to a malfunction in the generation of hydrogen

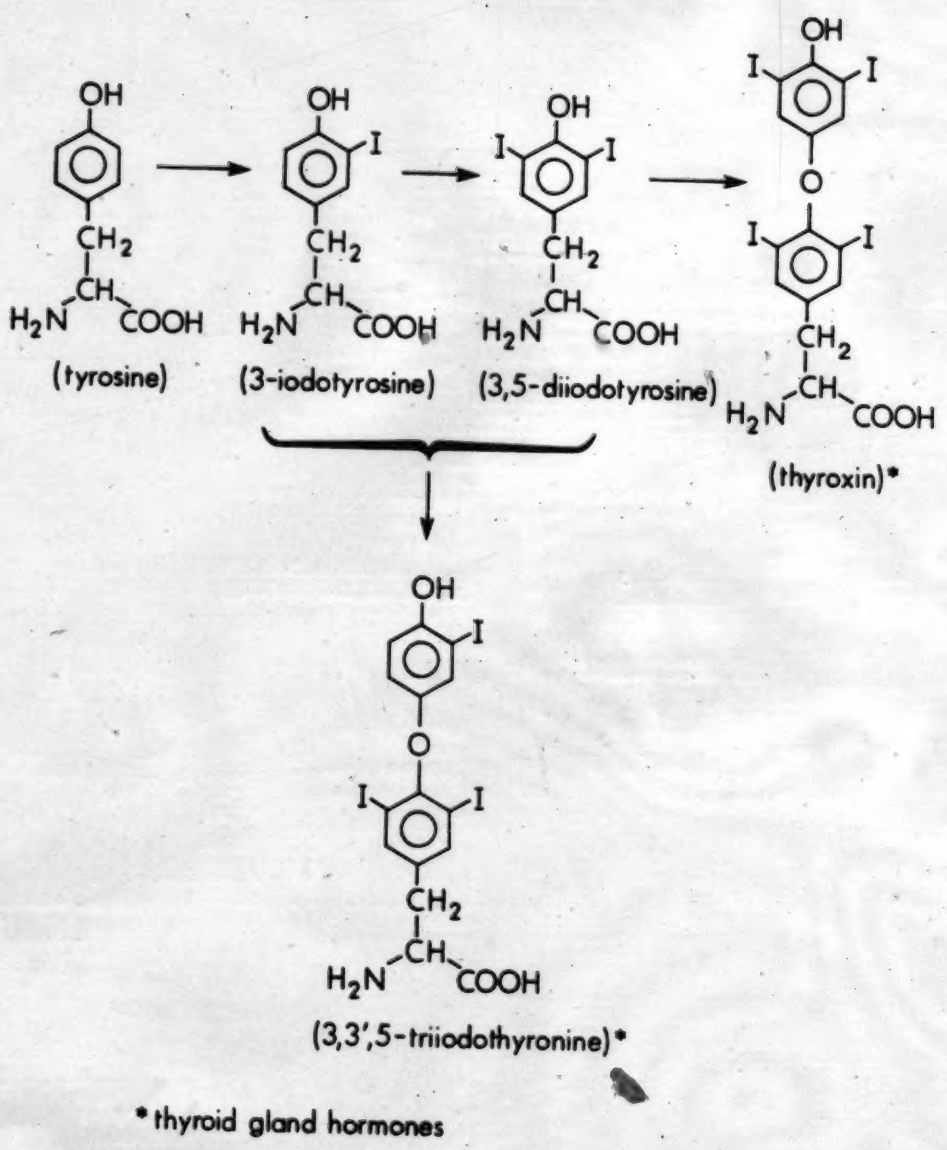


Fig. 1.5 Biosynthesis of thyroid gland hormones.

peroxide (66). Furthermore, in the treatment of hyperthyroidism, the antithyroid drugs which have been developed act as inhibitors of the thyroid peroxidase-catalyzed iodination (66).

1.4.1 Thyroid Peroxidase

The isolation of thyroid peroxidase in its native state has not yet been successfully carried out. The enzyme is tightly bound to cell membranes and is released only through proteolysis. This process undoubtedly alters the nature of the native peroxidase; however, active fragments have been isolated from both porcine (67-70) and bovine thyroids (71-74). The purest and most active fragment that has been isolated to date was obtained from hog thyroids (70). It has a molecular weight of 93,000 and is composed of two non-equivalent peptide chains. The carbohydrate content was estimated as 10%. Circular dichroism revealed approximately 20% each of α-helix and β-structure. The enzyme also has a large proline content. Thyroid peroxidase apparently contains heme; however, the exact nature of the heme group has not yet been elucidated. The most recent publication illustrates the spectral differences between hog thyroid and horseradish peroxidases and concludes that the heme components of these two enzymes are not identical (70). A study of beef thyroid peroxidase showed that the major fragment of molecular weight 92,000 also contains a heme group with similar properties as horseradish peroxidase (74).

Earlier studies on thyroid peroxidase using nonproteolytic methods of enzyme separation have yielded fragments of molecular weights as high as 450,000 (75). These preparations, however, exhibit less activity than the lower molecular weight fragments, indicating that parts of the cell membrane may still be attached (74). Nevertheless, the low molecular weight preparations may contain the active site and only part of the protein structure.

Four assays have been used to determine the activity of thyroid peroxidase preparations and may therefore signify the extent of purification (70). Iodide and guaiacol oxidations are easily followed spectrophotometrically. The incorporation of labelled iodide into bovine serum albumen may also be evaluated. The most complex assay involves the formation of I^{131}- labelled thyroxin from labelled thyroglobulin.

1.4.2 Tyrosine Iodination

Peroxidase-catalyzed iodination has been the subject of several investigations in recent years. In addition to the physiologically relevant iodination studies involving thyroid peroxidase and thyroglobulin, the oxidations of iodide and tyrosine by other peroxidases have also been studied in considerable detail.

The oxidation of iodide has long been recognized as one of the major steps in the iodination of tyrosine (37, 43,76,77). For the horseradish peroxidase-catalyzed reaction between iodide and hydrogen peroxide the iodide is

first oxidized to "$I^{\oplus}$" in a two-electron step from compound I to native enzyme without the formation of compound II (see Section 1.3.2, (37)). When released into solution, "$I^{\oplus}$" reacts very quickly with iodide to form molecular iodine which in turn reacts with excess iodide to yield triiodide ion. It was originally reported that horseradish peroxidase and iodine slowly form a complex (77); however, in a later study this complex was not observed spectrally (37). Reaction scheme II (Section 1.3.2) was established by titration experiments showing a 1:1 correspondence for the reactions of iodide with either compound I or compound II (37). Iodide oxidation by other peroxidases has also been investigated; compound II of lactoperoxidase forms a complex with iodide (78) and chloroperoxidase catalyzes the oxidation of iodide to iodate (79).

Tyrosine is readily oxidized by hydrogen peroxide in the presence of peroxidase to produce bityrosine (or dityrosine) linked at the position ortho to the hydroxyl group (80-83). By following the rate of bityrosine formation, a study of the kinetics of tyrosine oxidation catalyzed by both horseradish peroxidase and lactoperoxidase was carried out (83). The rate of product formation is maximized at pH 8.2 for catalysis by either enzyme. It was also discovered that the D and L isomers of tyrosine react with different rates (83,84); in the case of horseradish peroxidase, the reaction rate of the D isomer is double that of the L isomer at pH 8.2 (83). Myeloperoxidase catalyzes the oxidation of tyro-

sine, with the apparent formation of tyrosyl radicals (85).

Only the few forementioned studies have dealt separately with the peroxidase-catalyzed oxidations of iodide and of tyrosine. Other investigations have been centred upon the overall tyrosine iodination catalyzed by peroxidase, a more complex system that involves three substrates — hydrogen peroxide, iodide and tyrosine. Two mechanisms have been suggested for the formation of monoiodotyrosine (86): (a) enzymatic oxidations of iodide to molecular iodine and tyrosine to tyrosyl radical followed by a free radical reaction and, (b) oxidation of iodide to an "$I^{\oplus}$" species which reacts spontaneously with tyrosine.

The rate of tyrosine iodination and perhaps the reaction mechanism greatly depend upon the peroxidase and the pH. Nevertheless, a few researchers failed to recognize the significance of pH and drew general and misleading conclusions from experiments carried out at only one particular pH value. For example, one author surmised from a study at pH 7.4 that myeloperoxidase and thyroid peroxidase, but not horseradish peroxidase, were capable of catalyzing the formation of monoiodotyrosine (87). A later paper on horseradish peroxidase reported that the reaction of tyrosine with molecular iodine takes place nonenzymatically at pH 6.0 (88). It was finally observed that more meaningful results for the iodination of tyrosine may be obtained at low pH where the spontaneous reaction of molecular iodine with tyrosine is minimized. Using both lacto- and horse-

radish peroxidases, it was shown that at pH 3.6, the reaction of tyrosine with molecular iodine is indeed peroxidase-catalyzed (89). The rate of the overall reaction of tyrosine with iodide is maximized at pH ~ 5 using lactoperoxidase as a catalyst (90) and at pH ~ 4 for horseradish peroxidase catalysis (91). In addition, chloroperoxidase is successful in catalyzing the reaction of iodide with tyrosine; molecular iodine was observed as an intermediate and its subsequent reaction with tyrosine is also enzyme-catalyzed (92).

It has been postulated that horseradish peroxidase has two reaction sites with iodide binding to one and tyrosine to the other (91,93,94). The iodination is believed to occur on the enzyme surface either by the oxidations of tyrosine and iodide to free radical species which then couple, or by the oxidation of iodide to "$I^{\oplus}$" which reacts immediately with the bound tyrosine molecule. However, the binding of iodide to either horseradish peroxidase compound I or compound II has not been detected (37,43).

In a step towards the elucidation of the physiological reaction between iodide and the tyrosyl residues of thyroglobulin, the kinetics of iodination of several tyrosine-containing peptides has been studied (95-97). In an investigation involving thyroid, lacto- and horseradish peroxidases it was revealed that at pH 7.4, thyroid peroxidase is, in general, more efficient at catalyzing the iodination of tyrosyl peptides than either of the other two peroxid-

ases (95). In particular, the peptides containing Glu-Tyr were preferred by thyroid peroxidase (95); this is consistent with the amino acid composition of thyroglobulin (98). On the other hand, lactoperoxidase and horseradish peroxidase showed no preference towards these same peptides. In a separate study of the iodination of the peptide, Glu-Tyr-Glu, evidence was provided for the generation of a thyroid peroxidase - $I_{oxidized}$ species which subsequently reacts with the peptide (97).

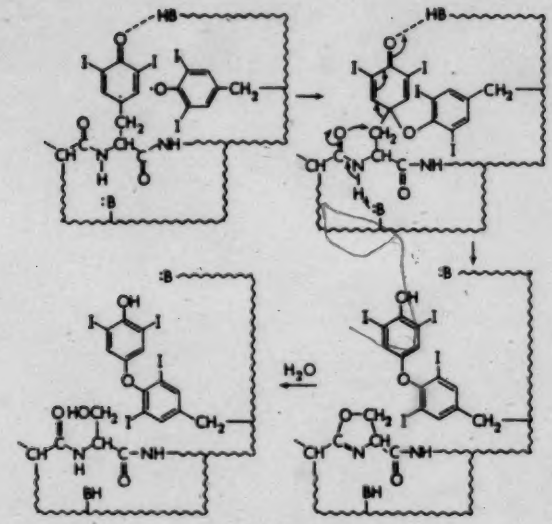
Thyroglobulin is the thyroid protein structure in which biological iodination and hormone synthesis takes place. For this reason, most of the biochemical studies with thyroid peroxidase involve thyroglobulin as substrate instead of free tyrosine (99-106). It was observed that excess iodide inhibits thyroglobulin iodination at pH values near neutrality. The competition of iodide with tyrosyl residues for a site(s) on the enzyme was given as one possible explanation (100,101). Excess diiodotyrosine also inhibits iodination, presumably for the same reason (103). Lactoperoxidase is as effective as thyroid peroxidase for the reaction of iodide with thyroglobulin at pH 7.0 (102). The pH optima for the thyroid, lacto- and horseradish peroxidase-catalyzed thyroglobulin iodinations are 7.5, 6.5 and 5.5 respectively (106).

The coupling of the iodo derivatives of thyroglobulin tyrosyl residues to form triiodothyronine and thyroxin (Fig. 1.5) is catalyzed by thyroid peroxidase (107). Two

mechanisms for the production of thyroxin are illustrated in Fig. 1.6 (65,86). Reaction mechanism V in Fig. 1.6 involves the peroxidase-catalyzed generation of free diiodotyrosyl radicals (108) within the thyroglobulin structure followed by intramolecular coupling. Alternatively, an intermolecular scheme was proposed (scheme VI, Fig. 1.6) whereby free diiodohydroxyphenylpyruvic acid hydroperoxide is produced via peroxidase catalysis; an ensuing reaction with thyroglobulin-bound diiodotyrosine yields thyroxin (109,110). There is not enough evidence to date to disprove either mechanism (66,111,112).

Other important factors influencing hormone production within the thyroglobulin matrix have been noted. It was observed that thyroxin formation takes place sequentially (113,114); tyrosine iodination is almost complete before coupling takes place (113). The concentration of free diiodotyrosine plays a significant role in controlling hormone production, although a clear explanation of this behaviour has not been presented (103,115). The structure of thyroglobulin and the spatial alignment of the iodotyrosines also affect the coupling reaction (104,116-119); this may be expected if mechanism V shown in Fig. 1.6 were operative. Studies with lactoperoxidase have shown its ability to catalyze the coupling reaction in thyroglobulin (102,120) and to form a weak complex with diiodotyrosine (121).

In Chapter IV the peroxidase-catalyzed reaction of iodide with tyrosine is discussed in greater detail. Because



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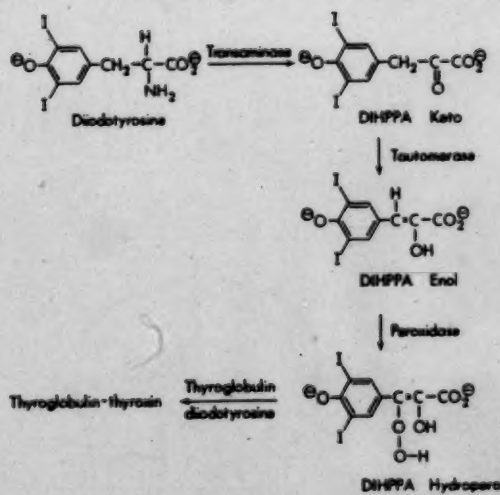

VI

Fig. 1.6 Proposed mechanisms for coupling of diiodotyrosine within thyroglobulin structure (65,86). DIHPPA = 4-hydroxy-3,5-diiodophenylpyruvic acid.

of the difficulty in isolating high purity thyroid peroxidase, horseradish peroxidase was used to study various aspects of tyrosine iodination. It is nevertheless impossible at the present time to make a direct correlation between the two enzymes since the properties of thyroid peroxidase have not yet been clearly defined. One should therefore bear in mind that the results presented in this thesis may not necessarily describe the physiological processes.

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CHAPTER II.

OXIDATIONS OF L-TYROSINE AND 3,5-DIIODO-L-TYROSINE BY COMPOUND I OF HORSERADISH PEROXIDASE

2.1 Summary

The rates of oxidation of both L-tyrosine and 3,5-diiodo-L-tyrosine with compound I of horseradish peroxidase were studied as a function of pH at 25°C and ionic strength 0.11. For the tyrosine-compound I reaction the effects of three ionizations were observed over the pH range 3.20 - 11.23. The pK_a values of the phenolic (pK_a ~ 10.1) and amino (pK_a ~ 9.2) dissociations of tyrosine and a single enzyme ionization (pK_a ~ 5.4) were determined by a nonlinear least squares analysis of the log (rate) versus pH profile. The reaction rate is maximized at pH 9.6 where only the phenolic group of tyrosine is protonated. An investigation of the diiodotyrosine-compound I reaction within the pH range 3.07 - 10.54 revealed the influences of one enzyme and two substrate ionizations. In order to explain the log (rate) versus pH profile, a mechanism was proposed whereby only the basic form of the enzyme (pK_a ~ 5.0) is reactive. The rate of oxidation is fastest at pH 5.8 with protonation of the phenolic group (pK_a ~ 6.6) of diiodotyrosine; nevertheless, the phenolate form remains reactive as long as the amino moiety (pK_a ~ 9.7) is protonated. Both tyrosine and diiodotyrosine reactions with compound I are described as base-catalyzed since it is the less acidic form of the en-

zyme which is most reactive. A total substrate charge of -1 is significant.

Tyrosine or diiodotyrosine is required in only a 0.5 M equivalent for the conversion of horseradish peroxidase compound I to compound II. The diiodotyrosine pK_a values were estimated as 6.4 and 9.4 for the phenolic and amino groups respectively.

2.2 Introduction

The oxidations of L-tyrosine and 3,5-diiodo-L-tyrosine by hydrogen peroxide in the presence of peroxidase are major steps in the elucidation of the physiologically relevant iodination and coupling reactions in the formation of thyroid gland hormones. Compound I of horseradish peroxidase contains both oxidizing equivalents of hydrogen peroxide and can react quickly with many substrates (1-9).

In this chapter, the redox reactions of compound I with tyrosine and diiodotyrosine are examined using pseudo-first order conditions over large pH ranges, and the kinetic parameters are analyzed. The pK_a values of the phenolic and amino groups of diiodotyrosine, determined independently by spectrophotometric and potentiometric measurements are also reported. In addition, this chapter includes an investigation of the stoichiometry of the tyrosine and diiodotyrosine reactions with compounds I and II.

2.3 Experimental Procedure

2.3.1 Materials

Horseradish peroxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase, lots 1366531, 1408333 and 1399140) was obtained from Boehringer-Mannheim Corp. as an ammonium sulphate precipitate, and was extensively dialyzed at 4°C against water distilled five times (9). The purity numbers (ratio of absorbances at 403 and 280 nm) were 3.28, 3.19 and 3.25 for lots 1366531, 1408333 and 1399140 respectively. The enzyme concentration was ascertained spectrophotometrically at 403 nm, using a molar absorptivity coefficient of 1.02×10^5 $M^{-1}cm^{-1}$ (10). Solutions of compound I were prepared by adding 0.9 M equivalents of hydrogen peroxide to the native enzyme; this ensured that none of the enzyme could be recycled. The standardization procedure for hydrogen peroxide has been described elsewhere (2).

Reagent grade L-(-)-tyrosine purchased from Eastman Kodak was used without further purification. Because of the difficulty in obtaining concentrated solutions of tyrosine, due to its relatively low solubility in water, stock solutions were prepared by dissolving the tyrosine in base, then titrating with acid. Standardized potassium hydroxide was first added to the partially dissolved tyrosine. After complete dissolution, the solution was neutralized with an equimolar quantity of nitric acid. The concentrations of resulting potassium and nitrate ions were 0.1 M. Recrystallization upon addition of acid did not present a problem

for solutions of 3 mM or less. For kinetic experiments at pH values less than 5 or greater than 10, concentrated nitric acid or potassium hydroxide was added to the stock tyrosine solutions until the desired pH was reached. This prevented pH fluctuations between dilute and concentrated sample solutions, where the buffer capacity was insufficient to counterbalance the effect of tyrosine ionizations.

3,5-Diiodo-L-tyrosine was obtained from Sigma Chemical Co. At the time of purchase the molecular weight was 463 as determined by carbon and nitrogen analysis (240 Elemental Analyzer). The discrepancy between this molecular weight and that of the anhydrous form (MW = 434) may be accounted for by the association of 1.6 water molecules per molecule of diiodotyrosine. Desiccation at -8°C prevented subsequent hydration. Diiodotyrosine solutions were prepared daily with heating to assist dissolution. The concentrations of stock solutions of both substrates were determined by weight, with an approximate error of 0.1%. Multidistilled water was used in the preparation of all solutions, since compound I reacts quickly with oxidizable impurities.

For the stopped-flow experiments, one of the drive syringes contained 1.0 μM compound I. Between pH values of about 5 and 9, buffer of ionic strength 0.01 was added to both substrate and enzyme. Outside of this pH range, however, only substrate contained buffer (ionic strength = 0.02) in order to prevent decomposition of enzyme in very acidic or basic solutions. Potassium nitrate was added to both

compound I and substrate solutions, maintaining a constant total ionic strength of 0.11. The estimated error in ionic strength is, at most, 5%.

2.3.2 Stopped-flow Method

The kinetic measurements involving tyrosine were carried out on a Gibson-Durrum stopped-flow spectrophotometer model D-110, equipped with a 2 cm cell; whereas, a Union-Giken stopped-flow spectrophotometer model RA-601 with a 1 cm cell was employed for the diiodotyrosine experiments. The two instruments have several different features. The Gibson-Durrum apparatus is provided with horizontal drive syringes, monochromatic light passing through the observation chamber and a stop plate on the stop syringe plunger. The Union-Giken model has vertical storage cells (excluding the problem of air bubbles entering the observation chamber), a monochromator positioned between the observation chamber and photomultiplier tube, and a stop valve with a variable duration of opening. The latter feature eliminates the need of drive plungers and a stop syringe, thereby simplifying the system.

The reactions were followed by monitoring the rate of disappearance of compound I at 411 nm, the isosbestic wavelength between native enzyme and compound II (see Fig. 1.2). Therefore, any ensuing reaction of compound II with the substrate to form the ferric enzyme was not observed. The solutions were thermostatted to maintain a temperature of

25.0 ± 0.1°C. The absorbance measurements were performed on Cary 14 and 219 spectrophotometers and pH was measured with a Fisher model 420 pH meter in conjunction with a Fisher combination electrode.

For the stopped-flow experiments, the substrate was in at least 10-fold excess of compound I to ensure pseudo-first order conditions. Between 7 and 11 traces of absorbance versus time were recorded for each reaction under each set of conditions. A typical pseudo-first order curve is illustrated in Fig. 2.1. The rate constant of each run was evaluated by a nonlinear least squares computer program of Gauss' method (11). The mean pseudo-first order rate constant, k_{obs}, and standard deviation were then determined. Plots of k_{obs} versus substrate concentration were constructed at selected pH values and the second order rate constants were obtained from the slopes of these plots. All other second order rate constants were calculated by dividing individual pseudo-first order rate constants by the corresponding concentration of substrate.

2.3.3 pK_a Values of Diiodotyrosine

Two methods, independent of the kinetic results, were used to determine the pK_a values of diiodotyrosine and, in each case, the experimental conditions were consistent with those of the stopped-flow procedure. For the spectrophotometric determination of the phenolic pK_a, the 2 ml sample contained 10^{-4}M diiodotyrosine. Citrate, phosphate and

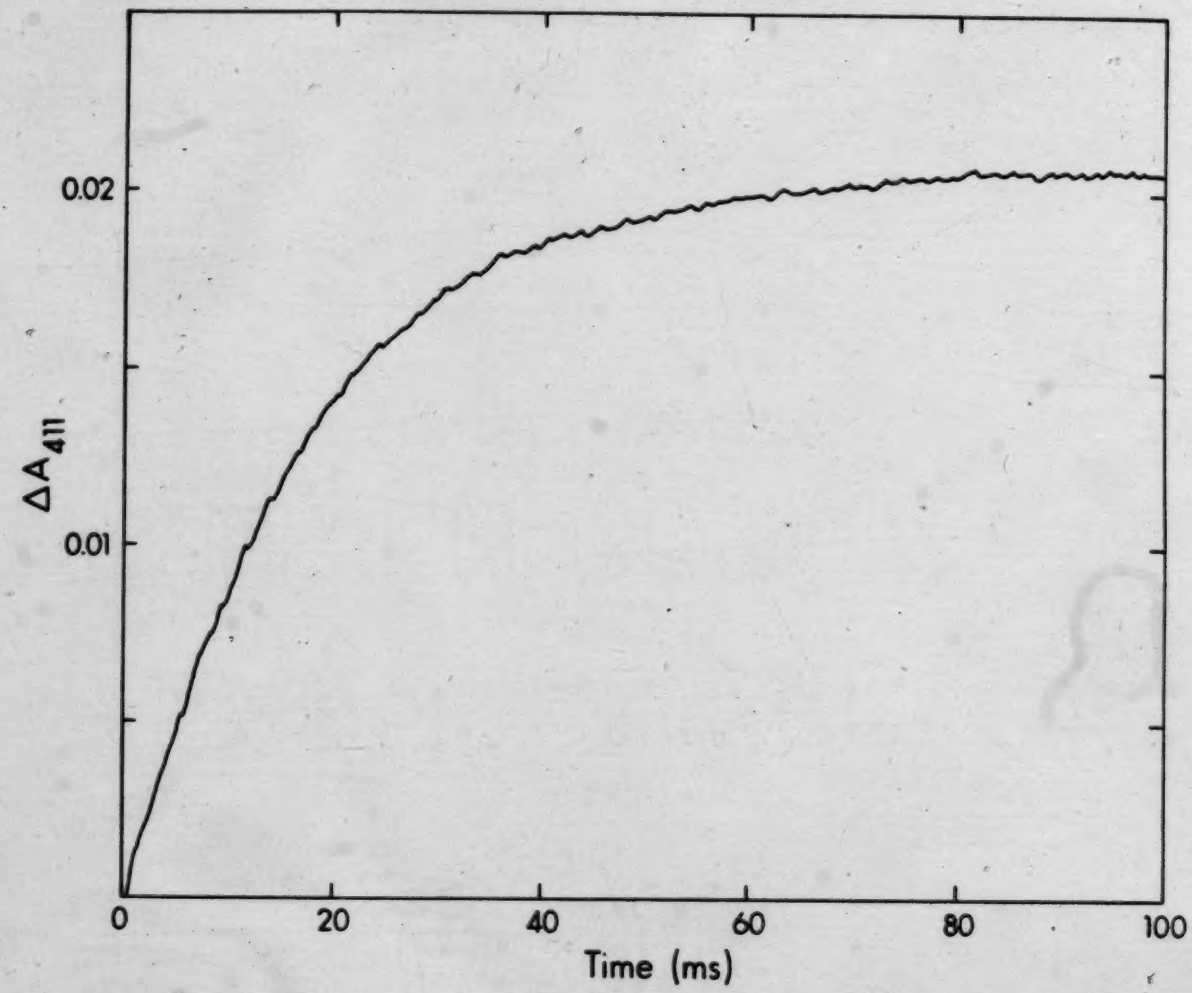


Fig. 2.1 Typical pseudo-first order trace at 411 nm for the reaction of horseradish peroxidase compound I with tyrosine. pH = 6.75, phosphate buffer. [compound I] = 0.5 μM and [tyrosine] = 5.72×10^{-4} M.

tris-HNO_3 buffers were employed, contributing 0.01 to the ionic strength. The addition of potassium nitrate resulted in a total ionic strength of 0.11. The reference and sample cuvettes contained identical concentrations of potassium nitrate and buffer. Absorbance was measured at 310 nm, the wavelength where the spectrum is most sensitive to pH changes.

For the determination of the approximate pK_a of the amino group of diiodotyrosine, a potentiometric titration was carried out. To 50 ml of 4.7×10^{-4} M diiodotyrosine (ionic strength = 0.10) increments of 0.095 M NaOH were introduced and the pH recorded. The maximum error in either the volume or ionic strength is 1%. For all diiodotyrosine pK_a experiments, the temperature was maintained at 25 ± 1°C.

2.3.4 Stoichiometry of the Substrate-Enzyme Reactions

Using a solution of 0.01 ionic strength tris-HNO_3 buffer at pH 8.4 and 0.10 ionic strength potassium nitrate, compound I was produced from the reaction of the ferric form of the enzyme with hydrogen peroxide. Compound II was then formed in 89% (or 93%) yield by the addition of a 0.5 M equivalent of tyrosine (or diiodotyrosine). Slow regeneration of the native enzyme resulted from a second 0.5 M equivalent addition of the substrate. These measurements were carried out on a Cary 219 spectrophotometer. The same concentrations of buffer and potassium nitrate were present

in the reference cuvette.

2.4 Results

2.4.1 pK_a Values of Diiodotyrosine

The pH dependence of the spectrum of diiodotyrosine is shown in Fig. 2.2. Two isosbestic points are evident at 270 and 290 nm and the greatest change in absorbance occurs at 310 nm. By measuring the absorbance change with pH at 310 nm an ionization of diiodotyrosine becomes evident as illustrated in Fig. 2.3. The following equation accounts for the absorbance dependence on pH.

$$[2.1] \qquad A = \left\{ \frac{\varepsilon_{SH} + \frac{\varepsilon_S \cdot K_S}{[H^+]}}{1 + \frac{K_S}{[H^+]}} \right\} [S]_o$$

In this equation, the derivation of which is given in Section 2.6, A corresponds to the absorbance, $[S]_o$ is the initial concentration of diiodotyrosine, K_S is the dissociation constant for the phenolic group of diiodotyrosine and ε_{SH} and ε_S are the molar absorptivity coefficients at 310 nm for the protonated and deprotonated forms due to this particular ionization. Using a nonlinear least squares program the value of K_S was found to be $(4.3 \pm 0.6) \times 10^{-7}$ ($pK_S = 6.37 \pm 0.06$) and the computed best-fit line is shown in Fig. 2.3.

Fig. 2.4 shows the potentiometric titration curve for diiodotyrosine. By simple calculation of the number of

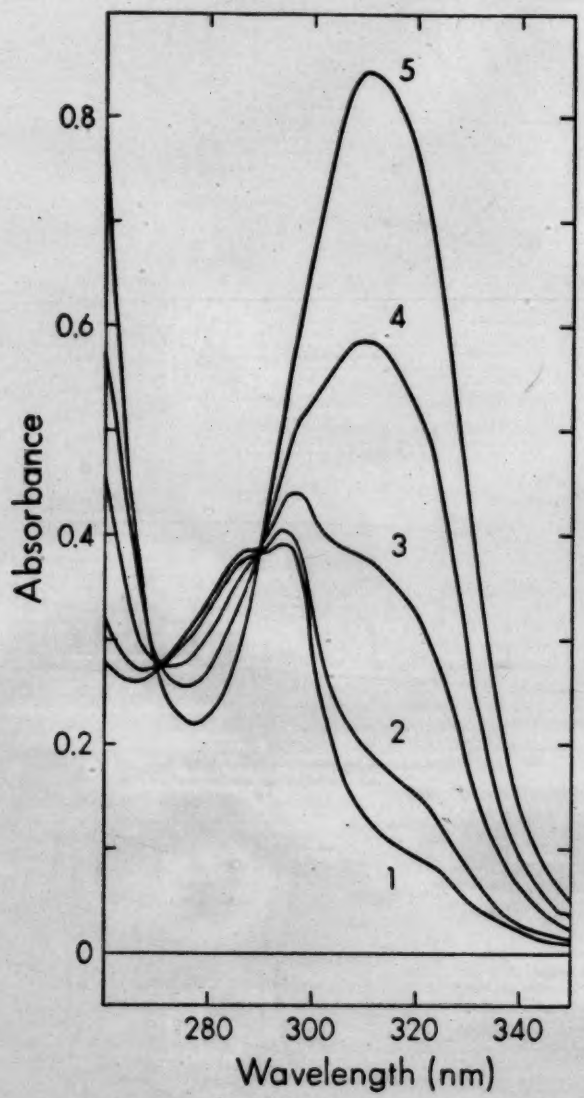


Fig. 2.2 pH dependence of diiodotyrosine spectra. All solutions are 0.10 ionic strength in KNO_3 and 0.01 ionic strength in buffer. Reference and sample cuvettes contain equal concentrations of KNO_3 and buffer. 1, pH = 5.40, citrate buffer; 2, pH = 5.68, citrate buffer, 3, pH = 6.14, phosphate buffer; 4, pH = 6.62, phosphate buffer; 5, pH = 9.40, 10.76 and 11.13, carbonate buffer.

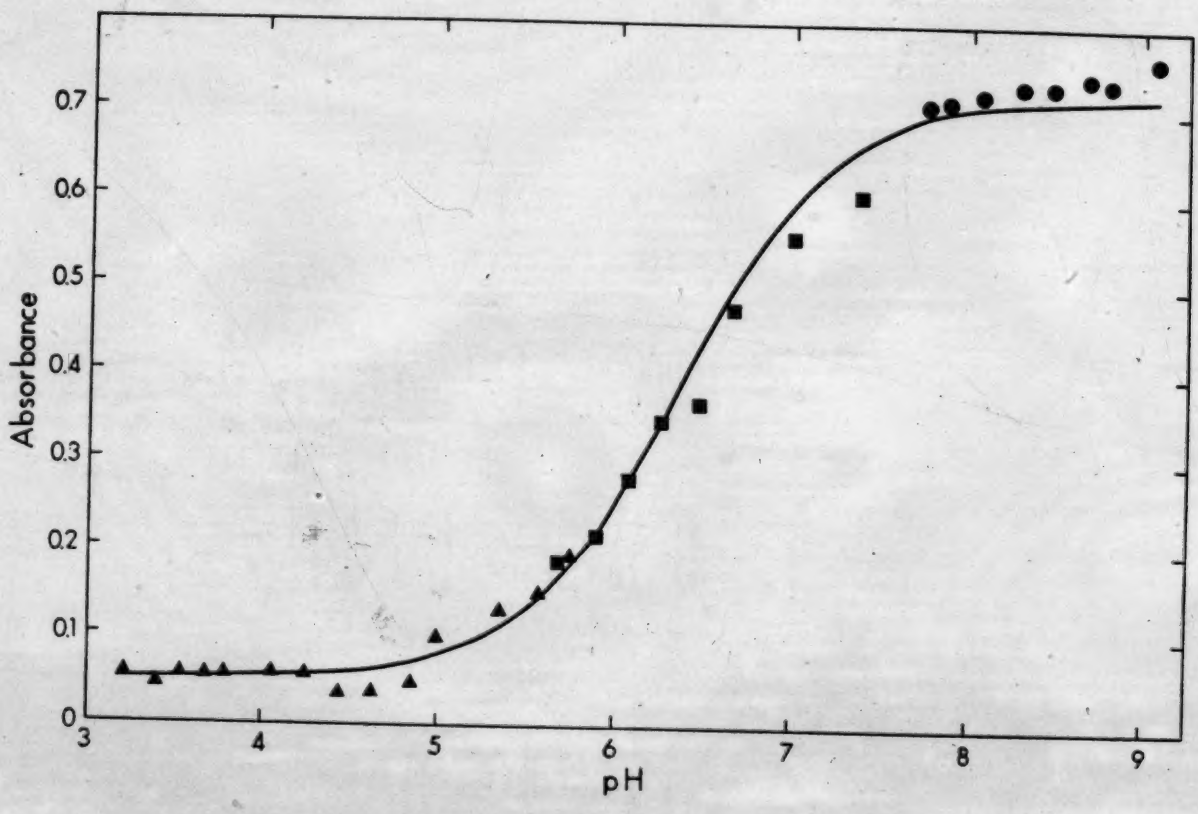


Fig. 2.3 Absorbance versus pH for diiodotyrosine at 310 nm. All solutions are 0.10 ionic strength in KNO_3 and 0.01 ionic strength in buffer. Reference and sample cuvettes contain equal concentrations of KNO_3 and buffer. ●, tris-HNO_3 buffer; ■, phosphate buffer; ▲, citrate buffer. The best-fit line was determined by a weighted nonlinear least squares analysis using Eq. 2.1.

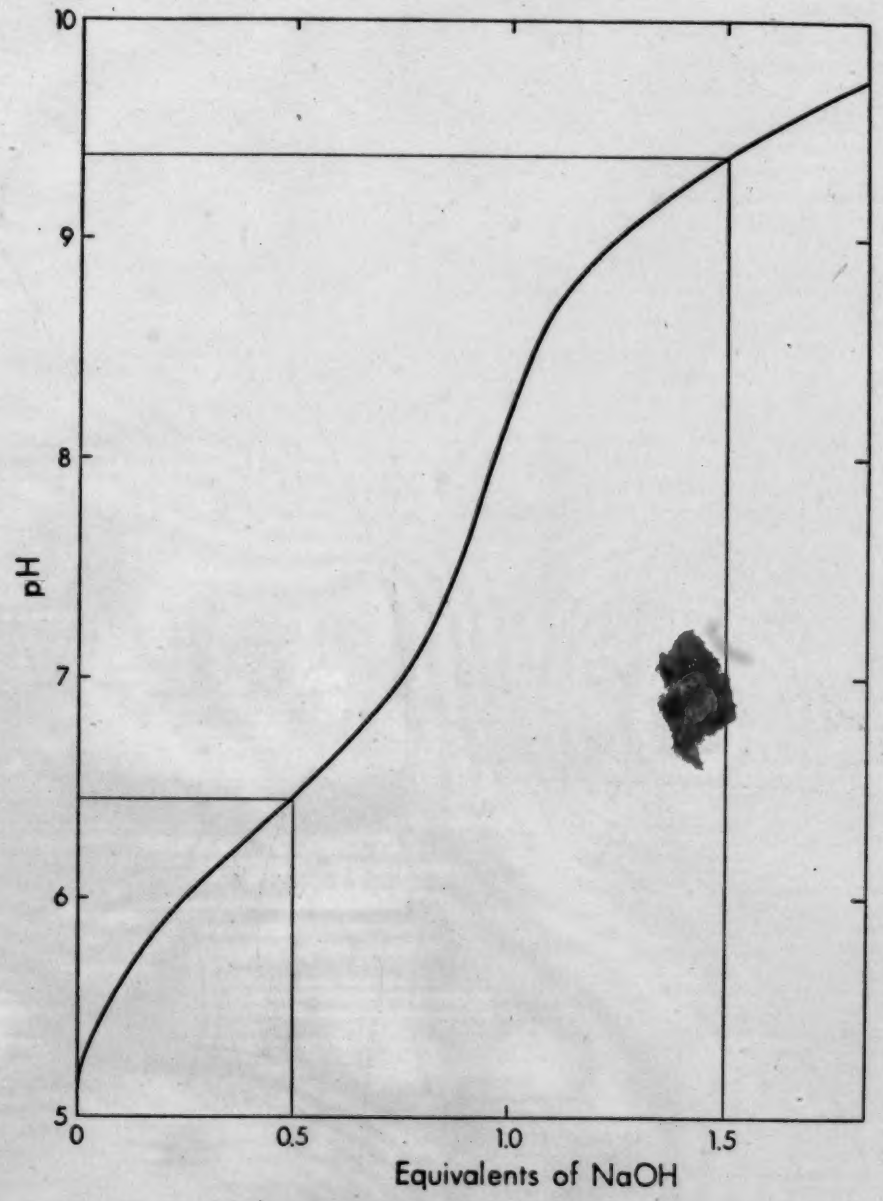


Fig. 2.4 Potentiometric titration curve for diiodotyrosine. The solution is 0.10 ionic strength in KNO_3 and 4.7×10^{-4}M diiodotyrosine. The volume is 50 ml and the temperature is 25 ± 1°C. The pK_a values for diiodotyrosine are indicated at 0.5 and 1.5 molar equivalents of NaOH.

base equivalents, the phenolic and amino pK_a values are read from the curve as 6.45 and 9.4 with estimated errors of ±0.05 and ±0.1 respectively. These values, as well as the phenolic pK_a of 6.37 determined spectrophotometrically are in agreement with several reported values (12-14). Nevertheless, a few inconsistencies in the literature (13,14) made this independent study necessary for clarification purposes.

2.4.2 Reactions of Compound I with Tyrosine and Diiodotyrosine

Typical plots of the observed rate constant, k_{obs}, versus substrate concentration are illustrated in Figs. 2.5 and 2.6. True second order kinetics is demonstrated by the linearity of each of these plots, obeying Eq. 2.2.

[2.2] $$k_{obs} = k_{app}[\text{substrate}]$$

The pH dependencies of k_{app}, the second order rate constant, for the reactions of compound I with tyrosine and diiodotyrosine are demonstrated in Figs. 2.7 and 2.8 respectively. The best-fit lines through the points were determined by a similar nonlinear least squares analysis used to determine k_{obs}. The data are compiled in Tables 2.1 and 2.2.

In order to determine the effect of nitrate, if any, at low pH, the compound I reaction with tyrosine was studied using potassium sulphate of ionic strength 0.10 and citrate

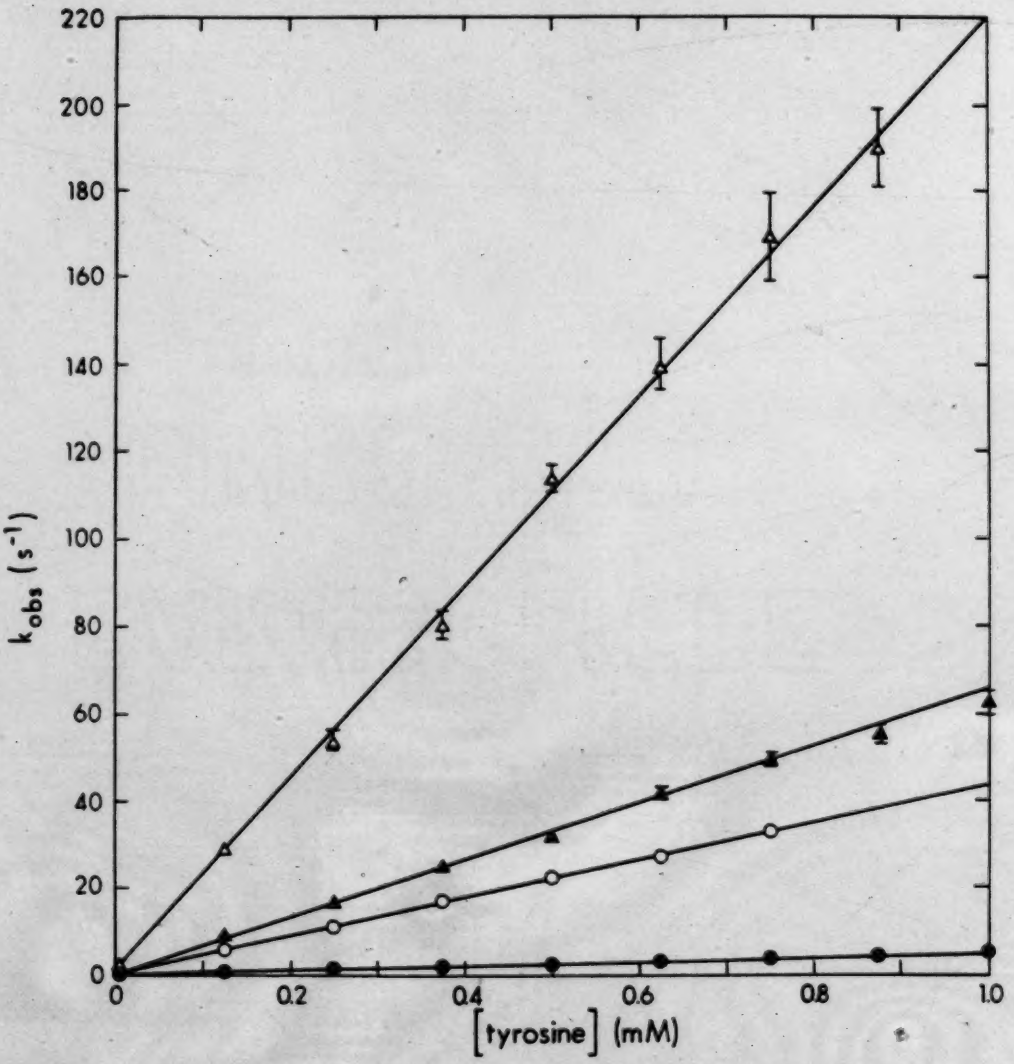


Fig. 2.5 Linear plots of k_{obs} versus [tyrosine] for the reaction of tyrosine with compound I. The slopes of the lines correspond to k_{app}, the second order rate constant, and are calculated by a weighted linear least squares analysis. The intercepts are equal to zero within the standard deviation. △, pH = 9.86, carbonate buffer; ▲ pH = 8.12, tris-HNO_3 buffer; ○, pH = 6.96, phosphate buffer; ● pH = 3.77, acetate buffer.

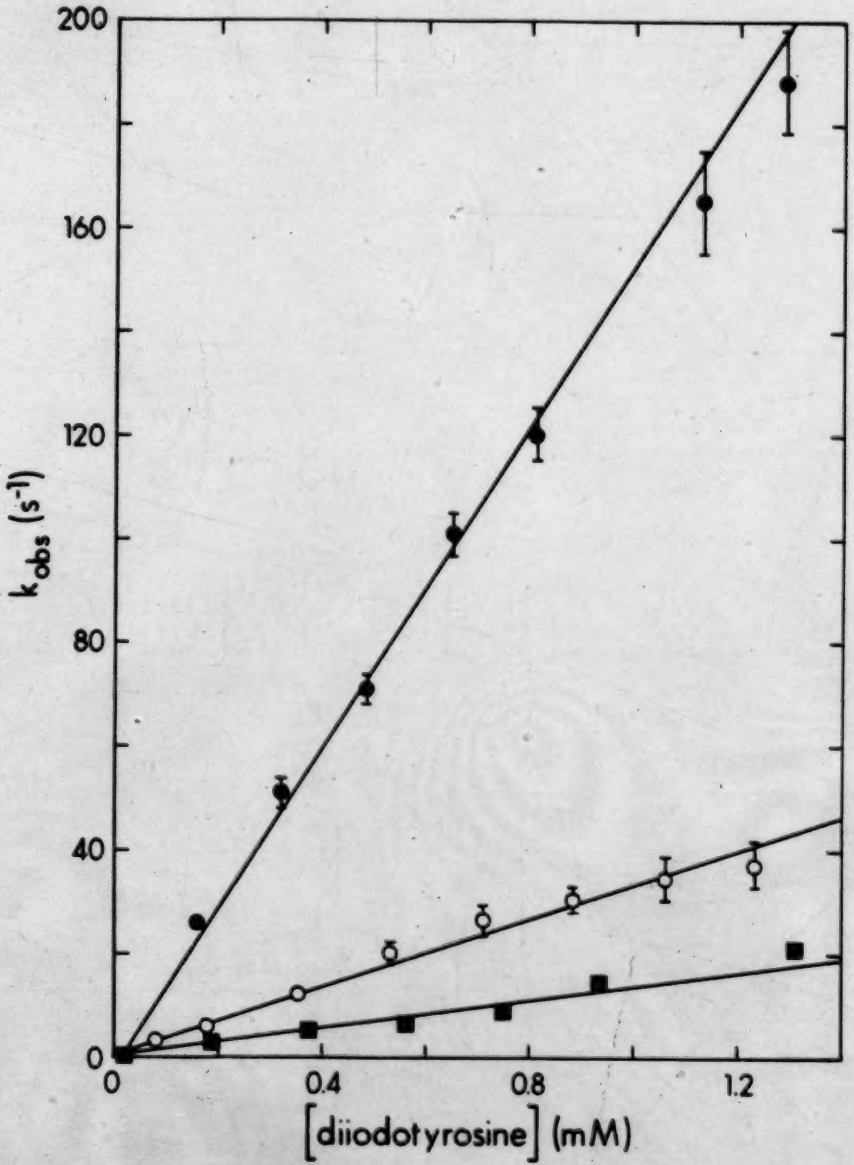


Fig. 2.6 Linear plots of k_{obs} versus [diiodotyrosine] for the reaction of compound I with diiodotyrosine. The slopes correspond to k_{app}, the second order rate constant. The intercepts which are zero within the standard deviation and the slopes are calculated by a weighted linear least squares analysis. ●, pH = 4.85, citrate buffer; ○, pH = 3.99, acetate buffer; ■, pH = 9.91, carbonate buffer.

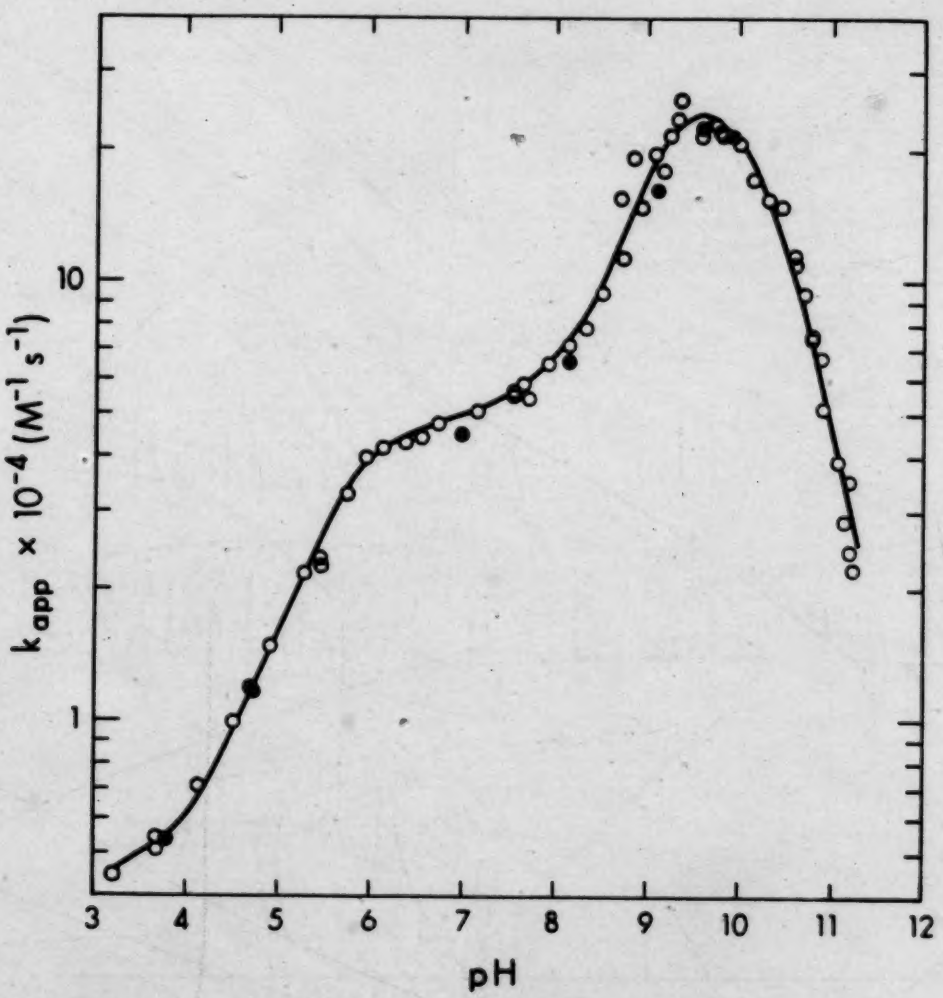


Fig. 2.7 Log (k_{app}) versus pH profile for the reaction of compound I with tyrosine. ●, k_{app} determined from slopes of plots of k_{obs} versus [tyrosine]. ○, k_{app} determined by dividing k_{obs} by appropriate [tyrosine]. The best-fit line was determined by weighted nonlinear least squares analysis using Eqs. 2.4 and 2.5.

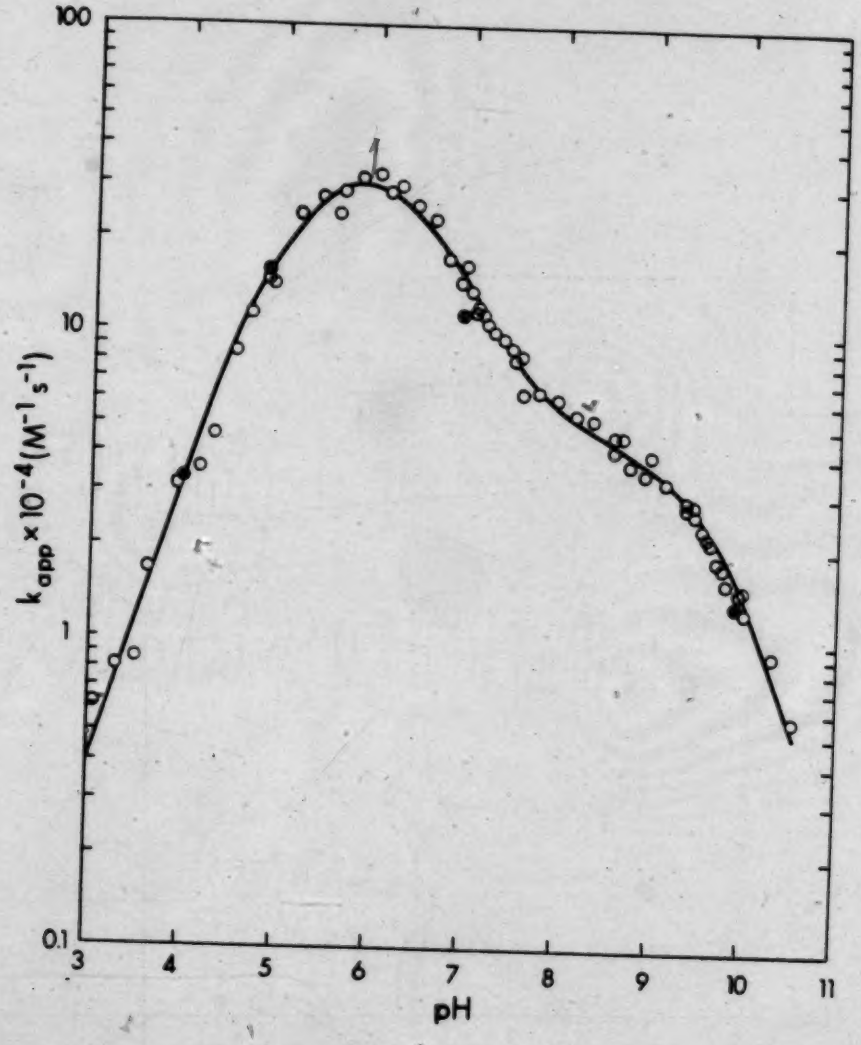


Fig. 2.8 Log (k_{app}) versus pH profile for the reaction of compound I with diiodotyrosine. ●, k_{app} determined from the slope of the plot of k_{obs} versus [diiodotyrosine]. ○, k_{app} determined by dividing k_{obs} by appropriate [diiodotyrosine]. The best-fit line was determined by weighted nonlinear least squares analysis using Eq. 2.7.

Table 2.1 Values of k_{app} as a Function of pH for the Reaction of Compound I and Tyrosine

pH	Buffer[a]	$k_{app} \times 10^{-4}$ ($M^{-1}s^{-1}$)[b]	[tyrosine] $\times 10^{3}$ (M)
3.20	CI	0.451 ± 0.008	0.25
3.68	CI	0.50 ± 0.02	0.25
3.69	CI	0.54 ± 0.01	0.25
3.77	A	0.532 ± 0.007[c]	0.005 - 1.00
4.12	CI	0.706 ± 0.009	0.25
4.52	CI	1.0 ± 0.1	0.25
4.71	CI	1.16 ± 0.03[c]	0.005 - 0.88
4.72	CI	1.18 ± 0.05	0.25
4.92	CI	1.49 ± 0.06	0.25
5.29	CI	2.2 ± 0.1	0.25
5.45	CI	2.64 ± 0.04	0.25
5.47	CI	2.5 ± 0.1	0.25
5.76	P	3.5 ± 0.1	0.25
5.95	P	3.95 ± 0.08	0.25
6.15	P	4.2 ± 0.1	0.25
6.37	P	4.3 ± 0.2	0.25
6.57	P	4.4 ± 0.2	0.25
6.75	P	4.8 ± 0.1	0.25
6.96	P	4.45 ± 0.05[c]	0.005 - 0.88
7.15	P	5.00 ± 0.09	0.25
7.52	P	5.5 ± 0.1	0.25
7.52	T	5.6 ± 0.2	0.25
7.63	P	5.8 ± 0.2	0.25
7.70	T	5.4 ± 0.3	0.25
7.91	T	6.5 ± 0.3	0.25
8.12	T	6.6 ± 0.3[c]	0.005 - 1.00
8.14	T	7.1 ± 0.6	0.25
8.31	T	7.8 ± 0.4	0.25
8.51	T	9.5 ± 0.3	0.25
8.67	C	15.7 ± 0.7	0.25
8.72	T	11.4 ± 0.6	0.25
8.80	C	19.2 ± 0.9	0.25
8.93	T	15.0 ± 0.5	0.25
9.05	C	20 ± 2	0.25
9.08	T	16.3 ± 0.6[c]	0.005 - 1.00
9.14	T	18 ± 1	0.25
9.22	C	22 ± 2	0.25
9.31	C	24 ± 2	0.25
9.33	C	26 ± 3	0.25
9.54	C	22.4 ± 0.3[c]	0.005 - 1.25
9.58	C	22 ± 1	0.25

Table 2.1, Continued

pH	Buffer[a]	$k_{app} \times 10^{-4}$ ($M^{-1}s^{-1}$)[b]	[tyrosine] $\times 10^{3}$ (M)
9.64	C	23 ± 2	0.25
9.68	C	23.3 ± 0.7	0.25
9.79	C	22 ± 2	0.25
9.80	C	22 ± 1	0.25
9.86	C	21.8 ± 0.3[c]	0.005 - 0.88
9.96	C	21 ± 1	0.25
10.12	C	17.4 ± 0.8	0.25
10.30	C	16 ± 1	0.25
10.42	C	15 ± 1	0.25
10.57	C	11.2 ± 0.5	0.25
10.58	C	11.6 ± 0.4	0.005
10.68	C	9.4 ± 0.2	0.25
10.73	C	7.3 ± 0.3	0.13
10.79	C	7.6 ± 0.3	0.25
10.84	C	6.8 ± 0.1	0.25
10.88	C	5.1 ± 0.1	0.25
11.05	C	3.9 ± 0.1	0.38
11.13	C	2.84 ± 0.07	0.63
11.16	C	3.49 ± 0.07	0.50
11.18	C	2.4 ± 0.1	0.75
11.23	C	2.22 ± 0.06	0.88

[a]Buffer Key: A, acetate; CI, citrate; P, phosphate; T, tris-HNO_3; C, carbonate.

[b]k_{app} determined by dividing the pseudo-first order rate constant by the appropriate tyrosine concentration, unless otherwise indicated. Error is the standard deviation of the mean value of k_{app}.

[c]k_{app} determined from the slope of the plot of k_{obs} versus [tyrosine]. Error is the standard deviation calculated by the least squares analysis.

Table 2.2 Values of k_{app} as a Function of pH for the Reaction of Compound I and Diiodotyrosine

pH	Buffer[a]	$k_{app} \times 10^{-4}$ ($M^{-1}s^{-1}$) [b]	[diiodotyrosine] $\times 10^{3}$ (M)
3.07	CI	0.61 ± 0.05	0.85
3.31	CI	0.8 ± 0.1	0.85
3.50	CI	0.9 ± 0.1	0.85
3.62	CI	1.7 ± 0.1	0.85
3.92	CI	3.2 ± 0.2	0.85
3.99	A	3.3 ± 0.1[c]	0.018 - 1.23
4.15	CI	3.6 ± 0.2	0.81
4.31	CI	4.6 ± 0.2	0.81
4.52	CI	8.5 ± 0.4	0.85
4.68	CI	11.6 ± 0.6	0.81
4.85	CI	14.9 ± 0.9	0.85
4.85	CI	15.6 ± 0.4[c]	0.016 - 1.29
4.90	CI	14.4 ± 0.6	0.85
5.17	CI	24 ± 2	0.93
5.39	CI	28 ± 1	0.81
5.58	CI	24 ± 2	0.81
5.63	CI	29 ± 2	0.81
5.81	CI	32 ± 1	0.81
5.99	P	33 ± 3	0.86
6.11	P	29 ± 7	0.86
6.23	P	30 ± 2	0.86
6.41	P	26 ± 3	0.86
6.60	P	24 ± 1	0.86
6.75	P	17 ± 1	0.86
6.90	P	14.7 ± 0.9	0.86
6.92	P	11.5 ± 0.6[c]	0.18 - 1.47
6.95	P	17 ± 1	1.41
7.02	P	13.8 ± 0.8	1.23
7.08	P	12 ± 2	1.06
7.09	P	12 ± 1	0.86
7.13	P	11.7 ± 0.6	0.88
7.19	P	11 ± 1	0.71
7.27	P	10.3 ± 0.8	0.53
7.37	P	9.7 ± 0.9	0.35
7.46	P	9.0 ± 0.5	0.18
7.49	P	8.3 ± 0.2	0.088
7.56	P	8.6 ± 0.6	0.018
7.58	T	6.4 ± 0.8	0.63
7.75	T	6.5 ± 0.3	0.63
7.96	T	6.2 ± 0.2	0.63
8.16	T	5.5 ± 0.5	0.63
8.34	T	5.4 ± 0.2	0.63

Table 2.2, Continued

pH	Buffer[a]	$k_{app} \times 10^{-4}$ ($M^{-1}s^{-1}$)[b]	[diiodotyrosine] $\times 10^{3}$ (M)
8.58	T	4.7 ± 0.2	0.63
8.58	C	4.2 ± 0.4	0.73
8.66	T	4.7 ± 0.3	0.63
8.73	C	3.8 ± 0.5	0.73
8.91	C	3.6 ± 0.5	0.73
8.97	T	4.1 ± 0.3	0.63
9.14	C	3.4 ± 0.1	0.73
9.36	C	2.8 ± 0.1	0.73
9.37	C	3.0 ± 0.2	0.98
9.44	C	2.9 ± 0.4	0.84
9.46	C	2.6 ± 0.1	0.73
9.53	C	2.4 ± 0.1	0.70
9.59	C	2.2 ± 0.3	0.56
9.62	C	2.2 ± 0.1	0.73
9.69	C	1.9 ± 0.1	0.42
9.75	C	1.8 ± 0.1	0.73
9.80	C	1.6 ± 0.2	0.28
9.91	C	1.36 ± 0.08[c]	0.019 - 1.31
9.93	C	1.5 ± 0.1	0.73
9.99	C	1.5 ± 0.1	0.73
10.02	C	1.29 ± 0.07	0.73
10.33	C	0.93 ± 0.07	0.93
10.54	C	0.57 ± 0.04	0.93

[a]Buffer Key: A, acetate; CI, citrate; P, phosphate; T, tris-HNO_3; C, carbonate.

[b]k_{app} determined by dividing the pseudo-first order rate constant by the appropriate diiodotyrosine concentration, unless otherwise indicated. Error is the standard deviation of the mean value of k_{app}.

[c]k_{app} determined from the slope of the plot of k_{obs} versus [diiodotyrosine]. Error is the standard deviation calculated by the least squares analysis.

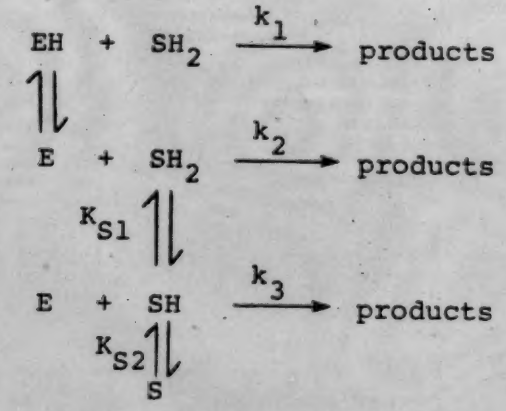
buffer of ionic strength 0.01 and pH 3.83. Similarly, for the compound I - diiodotyrosine reaction, the reaction was studied in the absence of nitrate using citrate buffer of ionic strength 0.11 at pH 4.16. The k_{app} values are $(5.2 \pm 0.5) \times 10^4\ M^{-1}\ s^{-1}$ and $(6.1 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$ for the compound I oxidations of tyrosine and diiodotyrosine respectively. These values are in good agreement with those obtained using nitrate to maintain a total constant ionic strength. Therefore, the rates of compound I reactions with either substrate are not influenced by nitrate and its possible binding to compound I (15).

2.5 Discussion

In the analysis of the log (k_{app}) versus pH profile, Fig. 2.7, two different substrate ionizations and a single enzyme ionization must be considered. In earlier work, the two tyrosine dissociation constants were determined spectrophotometrically to be 9.11 and 10.1 corresponding to the amino and phenolic groups respectively (16,17). Since the pK_a value of the carboxyl group lies outside the pH range of the study, the influence of the ionized carboxyl group is constant, and hence is not a factor in the analysis (18). The only other significant ionization, evident at pH ~ 5, must be due to the enzyme. It has previously been shown that an acid group on the enzyme with a $pK_a \sim 5$ has a pronounced effect on the kinetics of compound I reactions with other substrates (1-7), *p*-cresol included (9). A mechanism

which takes into account these three ionizations and which may explain the reaction sequence is shown in the following scheme

[2.3]



from which Eq. 2.4 can be derived.

$$[2.4] \quad k_{app} = \frac{\frac{k_1[H^+]}{K_E} + k_2 + \frac{k_3 K_{S1}}{[H^+]}}{\left(1 + \frac{[H^+]}{K_E}\right)\left(\frac{K_{S1}\,K_{S2}}{[H^+]^2} + \frac{K_{S1}}{[H^+]} + 1\right)}$$

In Eq. 2.4, the derivation of which is given in Section 2.6, K_{S1} and K_{S2} are the substrate ionization constants and K_E is the enzyme ionization constant; k_1, k_2 and k_3 are the second order rate constants for the most, intermediate and least protonated forms of the reactants.

The log (k_{app}) versus pH profile was first analyzed for pH values less than or equal to 6.37. This enabled a more precise determination of the enzyme pK_a, and simplified the computer analysis. For the pH range of 3.20 to 6.37, Eq. 2.5 takes into consideration only K_E, k_1 and k_2.

$$[2.5] \qquad k_{app} = \frac{k_1 + \frac{k_2 K_E}{[H^+]}}{1 + \frac{K_E}{[H^+]}}$$

The best-fit values for k_1, k_2 and K_E were determined by a nonlinear least squares analysis and are compiled in Table 2.3. Substitution of the numerical quantities of these constants into Eq. 2.4 and subsequent nonlinear least squares analysis yielded the remaining three parameters, k_3, K_{S1} and K_{S2}, which are also given in Table 2.3. It can be seen that the values of K_{S1} and K_{S2} determined from the kinetic experiments are in agreement with the published results (quoted above) which provides evidence that the analysis is correct. Also, the theoretical curve calculated on the basis of Eq. 2.4, gives an excellent fit to the experimental data in Fig. 2.7. On the other hand, kinetic results do not prove mechansims, they disprove them. What can be said with certainty is that any mechanism which leads to an equation which cannot fit the data is incorrect. Because of the complex nature of pH-rate profile and the strong evidence implicating the acid-base equilibria on tyrosine, there are few alternative equations with as few parameters as Eq. 2.4 which could fit the data.

The rate of reaction reaches a maximum at a pH value of 9.6. In this region, only the phenolic group of tyrosine is unionized. There is a rapid decrease in rate with either the protonation of the amino group or the deprotonation of

Table 2.3 Kinetic Parameters for the log (k_{app}) versus pH Profile for the Reaction of Compound I with Tyrosine

$k_1, M^{-1}s^{-1}$	$(4.5 \pm 0.1) \times 10^{3}$ [a]
$k_2, M^{-1}s^{-1}$	$(4.8 \pm 0.4) \times 10^{4}$ [a]
$k_3, M^{-1}s^{-1}$	$(4.0 \pm 0.6) \times 10^{5}$
K_E, M	$(3.8 \pm 0.3) \times 10^{-6}$ [a]
pK_E	5.42 ± 0.04
K_{S1}, M	$(6.1 \pm 0.9) \times 10^{-10}$
pK_{S1}	9.21 ± 0.07
K_{S2}, M	$(8 \pm 1) \times 10^{-11}$
pK_{S2}	10.10 ± 0.06

[a] Value determined from Eq. 2.5.

the phenolic group of the substrate. At low pH values, it is evident that the more acidic form of the enzyme is less reactive. The rate increases 10-fold between pH 3 and 6, due to an acid dissociation on the enzyme. Because it is the basic form of the enzyme that is most reactive, the reaction of compound I with tyrosine is described as base-catalyzed.

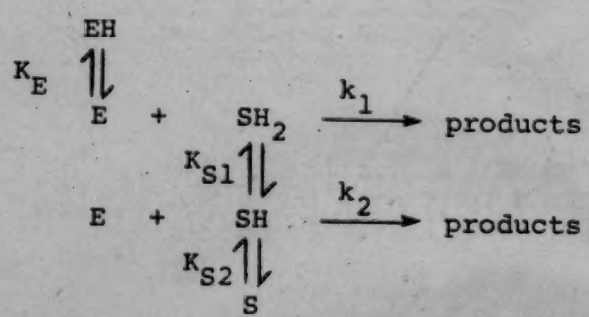
The oxidation of tyrosine has been previously studied using both lactoperoxidase and horseradish peroxidase as catalysts (19). Bayse *et al.* observed that lactoperoxidase was more effective than horseradish peroxidase at speeding up the rate of tyrosine oxidation with hydrogen peroxide; however, they found that the maximum reaction rate occurs at pH 8.2 for either enzyme. In the present study the rate maximum for the reaction of horseradish peroxidase compound I with tyrosine is observed at pH 9.6. It should be noted that Bayse's kinetic experiments were carried out by observing the formation of bityrosine, linked at the position ortho to the hydroxyl group of the phenyl. This tyrosine dimer had previously been isolated from the products of the reaction of tyrosine with hydrogen peroxide in the presence of horseradish peroxidase (20).

It is interesting that tyrosine resembles *p*-cresol in that the reaction of horseradish peroxidase with either substrate results in dimerization. Another similarity is that base catalysis is exhibited when either substrate reacts with compound I (9). Both tyrosine and *p*-cresol are most

reactive when the phenolic group is unionized, and when the enzyme is in its most basic form. One major difference in the two reactions is, however, the considerably slower reaction rate of tyrosine with compound I. For other substrates, such as iodide ion (7), ferrocyanide ion (1-3), bisulphite ion (6), and *p*-aminobenzoic acid (4,5), acid catalysis has been demonstrated. The rates of reaction of compound I with those substrates are comparable with the tyrosine reaction rate.

The dependence of k_{app} on pH for the oxidation of diiodotyrosine by compound I is shown in Fig. 2.8. The best-fit line was calculated by a computer analysis of the following mechanism:

[2.6]



EH and E are the acidic and basic forms of the enzyme, with K_E the corresponding equilibrium constant. SH_2, SH and S are the least, intermediate and most basic forms of diiodotyrosine. K_{S1} and K_{S2} are respectively the equilibrium constants for the phenolic and amino group ionizations. The carboxyl group of diiodotyrosine remains essentially unprotonated and changes in reaction rate in the pH range of

study should not be affected by this ionization (18). k_1 and k_2 are the second order rate constants for the reactions involving the phenolic and phenolate forms of diiodotyrosine. The least squares values for the five parameters in scheme 2.6 are given in Table 2.4. These were calculated using Eq. 2.7, the derivation of which is given in Section 2.6.

$$[2.7] \qquad k_{app} = \frac{\frac{k_1[H^+]}{K_{S1}} + k_2}{\left(1 + \frac{[H^+]}{K_E}\right)\left(\frac{K_{S2}}{[H^+]} + 1 + \frac{[H^+]}{K_{S1}}\right)}$$

On the basis of mechanism 2.6 and Eq. 2.7, the calculated pK_a values for diiodotyrosine of 6.6 and 9.7 are in reasonable agreement with those determined spectrophotometrically and potentiometrically. This correlation and the good fit of the theoretical curve through the data points in Fig. 2.8 strongly suggest that the analysis is accurate. This does not exclude other possibilities; however, it would be difficult to find a simpler mechanism to describe the behaviour.

One of the primary features of the log (rate) versus pH profile is an initial rate increase at low pH due to an enzyme acid group ionization of calculated pK_a 5.0. It has been noted that this same group, with a pK_a value ranging between 4.6 and 5.4, has an effect on the reactions of compound I with other substrates (1-7,9). In the case of the oxidation of diiodotyrosine by compound I, the reaction may

Table 2.4 Kinetic Parameters for the log (k_{app}) versus pH Profile for the Reaction of Compound I with Diiodotyrosine

k_1, $M^{-1}s^{-1}$	$(4.0 \pm 0.9) \times 10^{5}$
k_2, $M^{-1}s^{-1}$	$(4.4 \pm 0.2) \times 10^{4}$
K_E, M	$(9.2 \pm 0.9) \times 10^{-6}$
pK_E	5.04 ± 0.04
K_{S1}, M	$(2.6 \pm 0.4) \times 10^{-7}$
pK_{S1}	6.58 ± 0.07
K_{S2}, M	$(2.2 \pm 0.2) \times 10^{-10}$
pK_{S2}	9.66 ± 0.05

be described as base-catalyzed since the rate is greatly enhanced with deprotonation of this enzyme group. The same observation is noted for compound I reactions with *p*-cresol (9) and tyrosine.

The rate of diiodotyrosine oxidation reaches a maximum at pH 5.8. In this pH region, the basic form of the enzyme and the phenolic (not phenolate) form of substrate are present in largest concentrations. The rate falls rapidly with either protonation of the enzyme or removal of the phenolic proton. This may be explained in terms of oxidation taking place via hydrogen atom transfer from the phenolic group of diiodotyrosine to the deprotonated enzyme group. The behaviour of the compound I reactions with *p*-cresol (9) and tyrosine may be interpreted in the same way. Similarly, for the oxidation of *p*-cresol by compound II of horseradish peroxidase, it was the phenolic, and not the phenolate, form of the substrate which was most reactive (21). The enzyme acid group of $pK_a \sim 5$ also influences the compound I reactions with ferrocyanide (1-3), *p*-aminobenzoic acid (4,5), iodide (7) and bisulphite (6). For these substrates, however, the protonated enzyme form is most reactive; possibly electron transfer, unaccompanied by proton transfer, from the substrate to the enzyme is facilitated.

Quite unexpectedly, the effect of ionization of the amino moiety of diiodotyrosine on the rate of oxidation is opposite to that observed for the compound I - tyrosine reaction. In the case of tyrosine as substrate, the rate

increases with dissociation of the amino group; whereas, for the diiodotyrosine reaction with compound I, the oxidation is faster with protonation of this same group. In order to explain this contradiction, the total substrate charge should be considered. First of all, the most reactive form of tyrosine has a total charge of -1 ($-OH, -NH_2, -COO^{\ominus}$). The neutral molecule ($-OH, -NH_3^{\oplus}, -COO^{\ominus}$) will also react, but, at a much slower rate. No reaction is observed between compound I and the most basic form of tyrosine of total charge -2 ($-O^{\ominus}, -NH_2, -COO^{\ominus}$). In this instance, it appears that protonation of the tyrosine phenolic group is a required condition for reaction. Secondly, the rate increases when the total substrate charge is -1. For the reaction of compound I with diiodotyrosine, the neutral substrate with a protonated phenolic group ($-OH, -NH_3^{\oplus}, -COO^{\ominus}$) is most reactive. Nevertheless, reaction takes place, albeit at a slower rate, with dissociation of the proton from this group, as long as the amino moiety is protonated and the total charge on the diiodotyrosine is -1 ($-O^{\ominus}, -NH_3^{\oplus}, -COO^{\ominus}$). Further deprotonation of the amino group, giving the substrate a charge of -2 ($-O^{\ominus}, -NH_2, -COO^{\ominus}$) results in no reactivity. For the diiodotyrosine reaction with compound I, it appears that the most important factor is, once again, protonation of the phenolic group; the second consideration is the total charge on the substrate. If a positive charge lies near the compound I active site for molecules such as tyrosine or diiodotyrosine, then the reaction with either substrate

carrying a total negative charge should be favoured. In the case of the singly-charged diiodotyrosine molecule, a proton may be transferred from the amino moiety to the enzyme group of pK_a 5.0 in order to initiate the electron transfer from the phenolate anion. Neither the tyrosine nor diiodotyrosine of charge -2 is reactive due to the lack of protons available for transfer.

The preceding information may suggest that the oxidation of tyrosine and its iodo derivative takes place at a site very close to the ferryl ion. Studies carried out on the binding of *p*-cresol (22,23), benzhydroxamic and indolepropionic acids (23) to the native enzyme have suggested that binding occurs at a site very close to the heme. Although there is no evidence that tyrosine and diiodotyrosine form complexes with compound I, they may very well react at a position similar to the binding site of *p*-cresol on the ferric enzyme. It should also be taken into consideration that a π-cation radical may attract substrates of a single negative charge (24). Future investigations using new and varied substrates may clarify the rate dependence of compound I reactions on substrate charge.

An interesting feature of the interactions of tyrosine and diiodotyrosine with the intermediates of horseradish peroxidase is the formation of compound II from compound I by a 0.5 M equivalent of either substrate. A subsequent 0.5 M equivalent of the substrate is sufficient to reduce compound II to the native enzyme. This same stoichiometry

was observed for *p*-cresol (Section 1.3.2, (25)). The direct oxidation by hydrogen peroxide of either *p*-cresol or tyrosine causes dimerization. The product of diiodotyrosine oxidation may also be a dimer; however, it is difficult to speculate where exactly the coupling may occur.

2.6 Derivations of Eqs. 2.1, 2.4 and 2.7

Derivation of Eq. 2.1:

$$A = \varepsilon_S[S] + \varepsilon_{SH}[SH]$$

$$K_S = \frac{[S][H^+]}{[SH]}$$

$$A = \left(\frac{\varepsilon_S K_S + \varepsilon_{SH}[H^+]}{K_S}\right)[S]$$

$$[S]_o = [S] + [SH]$$

$$A = \left(\frac{\varepsilon_S K_S + \varepsilon_{SH}[H^+]}{K_S}\right)\left(\frac{[S]_o}{1 + \frac{[H^+]}{K_S}}\right)$$

$$A = \left(\frac{\varepsilon_{SH} + \frac{\varepsilon_S K_S}{[H^+]}}{1 + \frac{K_S}{[H^+]}}\right)[S]_o$$

Derivation of Eq. 2.4:

$$v = k_{app}\,[E]_{total}\,[S]_{total} = k_1[EH][SH_2] + k_2\,[E][SH_2] + k_3\,[E][SH]$$

$$[E]_{total} = [EH] + [E]$$

$$[S]_{total} = [SH_2] + [SH] + [S]$$

$$k_{app} = \frac{k_1[EH][SH_2] + k_2[E][SH_2] + k_3[E][SH]}{([EH]+[E])\ ([SH_2]+[SH]+[S])}$$

$$k_{app} = \frac{\dfrac{k_1[H^+]}{K_E} + k_2 + \dfrac{k_3K_{S1}}{[H^+]}}{\left(1 + \dfrac{[H^+]}{K_E}\right)\left(\dfrac{K_{S1}K_{S2}}{[H^+]^2} + \dfrac{K_{S1}}{[H^+]} + 1\right)}$$

Derivation of Eq. 2.7:

$$v = k_{app}\,[E]_{total}\,[S]_{total} = k_1[E][SH_2] + k_2\,[E][SH]$$

$$[E]_{total} = [E] + [EH]$$

$$[S]_{total} = [S] + [SH] + [SH_2]$$

$$k_{app} = \frac{k_1[E][SH_2] + k_2[E][SH]}{([E]+[EH])([S]+[SH]+[SH_2])}$$

$$k_{app} = \frac{\dfrac{k_1[H^+]}{K_{S1}} + k_2}{\left(1 + \dfrac{[H^+]}{K_E}\right)\left(\dfrac{K_{S2}}{[H^+]} + 1 + \dfrac{[H^+]}{K_{S1}}\right)}$$

2.7 Acknowledgement

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CHAPTER III.

OXIDATIONS OF L-TYROSINE AND 3,5-DIIODO-L-TYROSINE BY COMPOUND II OF HORSERADISH PEROXIDASE

3.1 Summary

The oxidations of both L-tyrosine and 3,5-diiodo-L-tyrosine by compound II of horseradish peroxidase were studied over the pH range of approximately 3 to 10 at 25°C and at a constant ionic strength of 0.11. The log(rate) versus pH profile for the tyrosine-compound II reaction illustrates the influences of at least two acid group ionizations. An enzyme dissociation ($pK_a \sim 6.2$) has a small effect on the reaction rate; whereas, a second pK_a of 9.2, which may be attributed to either the enzyme or substrate, has a greater influence on the rate. The oxidation of tyrosine by compound II is fastest at pH 7.6. In the case of the diiodotyrosine-compound II reaction, three acid dissociations are necessary to describe the plot of log (k_{app}) versus pH. These include two enzyme pK_a values of 3.6 and 8.6, and one substrate pK_a of 6.6. The rate optimum for the reaction occurs at pH 5.2 and deprotonation of the phenolic group of diiodotyrosine results in a dramatic decrease in k_{app}.

3.2 Introduction

Compound II is the intermediate species of horseradish peroxidase which contains only one of the two oxidizing equivalents of hydrogen peroxide. The pH dependencies of the oxidations of various substrates by compound II have been

studied in detail (1-9, Fig. 1.4). Compound II reactions are generally slower than their counterpart compound I reactions.

The reaction rates and corresponding mechanisms for the oxidations of L-tyrosine and 3,5-diiodo-L-tyrosine by compound II of horseradish peroxidase are discussed in this chapter. These studies were conducted over wide pH ranges in order to evaluate the kinetic and equilibrium parameters.

3.3 Experimental Procedure

3.3.1 Materials

Horseradish peroxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase, lot 1227335) was purchased from Boehringer-Mannheim Corp. as an ammonium sulphate suspension. After extensive dialysis in water distilled five times (10), the concentration of the enzyme solution was determined spectrophotometrically at 403 nm using a molar absorptivity coefficient of 1.02×10^5 $M^{-1}cm^{-1}$ (11). The purity number (ratio of absorbances at 403 and 280 nm) was 3.31. Compound II was prepared by subsequent additions of a 1.1 M equivalent of potassium ferrocyanide and a 0.9 M equivalent of hydrogen peroxide. The slight excess of ferrocyanide ensured complete conversion of compound I to compound II; whereas, the less-than-equivalent amount of hydrogen peroxide prevented recycling of the enzyme. Concentrations of ferrocyanide and hydrogen peroxide were respectively determined by weight and by use of horseradish peroxidase as catalyst for I_3^- production from I^- (2).

L-(-)-tyrosine was obtained from Eastman Kodak and recrystallized from multi-distilled water. Fresh solutions were prepared daily with heating to assist dissolution. Precipitation upon cooling posed no problem for concentrations less than 3 mM. 3,5-Diiodo-L-tyrosine was bought from Sigma Chemical Co. In order to prevent further hydration, diiodotyrosine was stored in a desiccator at -8°C. Like tyrosine, the solubility of diiodotyrosine was enhanced by heating. The concentrations of the stock solutions of both substrates were determined by weight, with an error of ~0.1%.

3.3.2 Stopped-Flow Method

For all stopped-flow experiments a constant total ionic strength of 0.11 was maintained for the final solutions. Both enzyme and substrate solutions were always 0.10 ionic strength in potassium nitrate. For pH values between 5 and 9, 0.01 ionic strength buffer was added to both enzyme and substrate; however, outside of this pH range, where the chance of enzyme decomposition increases, only the substrate solutions contained buffer (ionic strength = 0.02). In all cases, the concentration of compound II in the mixing chamber was 0.5 μM. The concentrations of the substrate solutions were in at least a 10-fold excess of the enzyme, ensuring pseudo-first order conditions.

All kinetic measurements were performed on a Union Giken stopped-flow spectrophotometer model RA-601 equipped

with a thermostatted 1 cm cell. The rate of disappearance of compound II was followed at 425 nm, the wavelength of its maximum absorbance. Between 7 and 11 traces were recorded for each specific reaction, with subsequent evaluation of the mean rate constant and its standard deviation. All solutions were maintained at 25.0 ± 0.1°C. A Cary 219 spectrophotometer was used for the absorbance measurements and the pH was monitored by a Fisher model 420 pH meter attached to a Fisher combination electrode.

3.4 Results

The relationship between the observed rate constant, k_{obs}, and substrate concentration is demonstrated in Figs. 3.1 and 3.2. If true pseudo-first order kinetics is obeyed then the linear plot with a zero-intercept may be described by Eq. 3.1.

$$[3.1] \qquad k_{obs} = k_{app}[\text{substrate}]$$

The apparent second order rate constant, k_{app}, is calculated from the slope.

The best-fit plots of log (k_{app}) versus pH for the reactions of tyrosine and diiodotyrosine with compound II are given in Figs. 3.3 and 3.4 and the experimental rate data are summarized in Tables 3.1 and 3.2. In both cases, the closed circles represent values of k_{app} which were determined from the slopes of k_{obs} versus [substrate] plots; whereas, the open circles are each the result of single experiments in which k_{app} is obtained by dividing k_{obs} by the

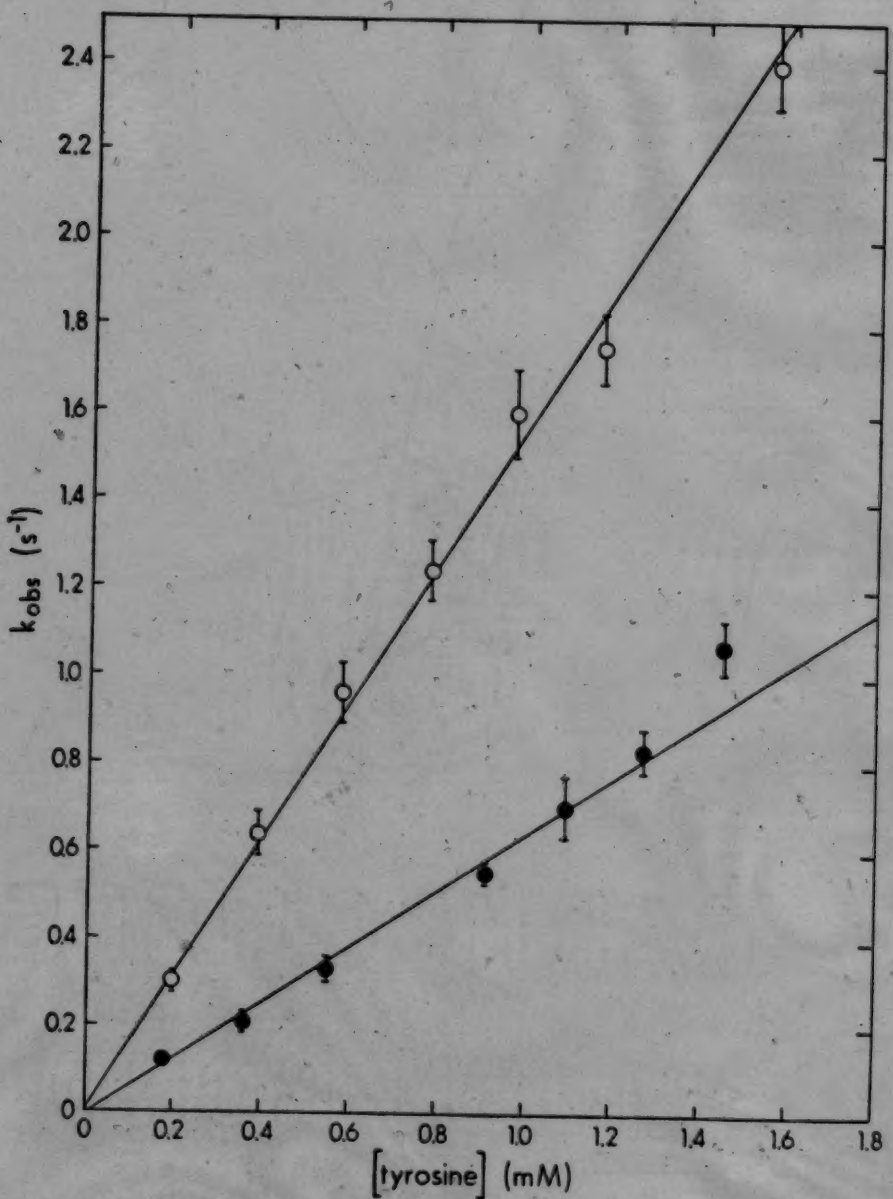


Fig. 3.1 Linear plots of k_{obs} versus [tyrosine] for the reaction of compound II with tyrosine. The slopes correspond to k_{app}, the second order rate constant. The intercepts which are zero within the standard deviation and the slopes are calculated by a weighted linear least squares analysis. ●, pH = 5.10, phosphate buffer; O, pH = 7.68, phosphate buffer.

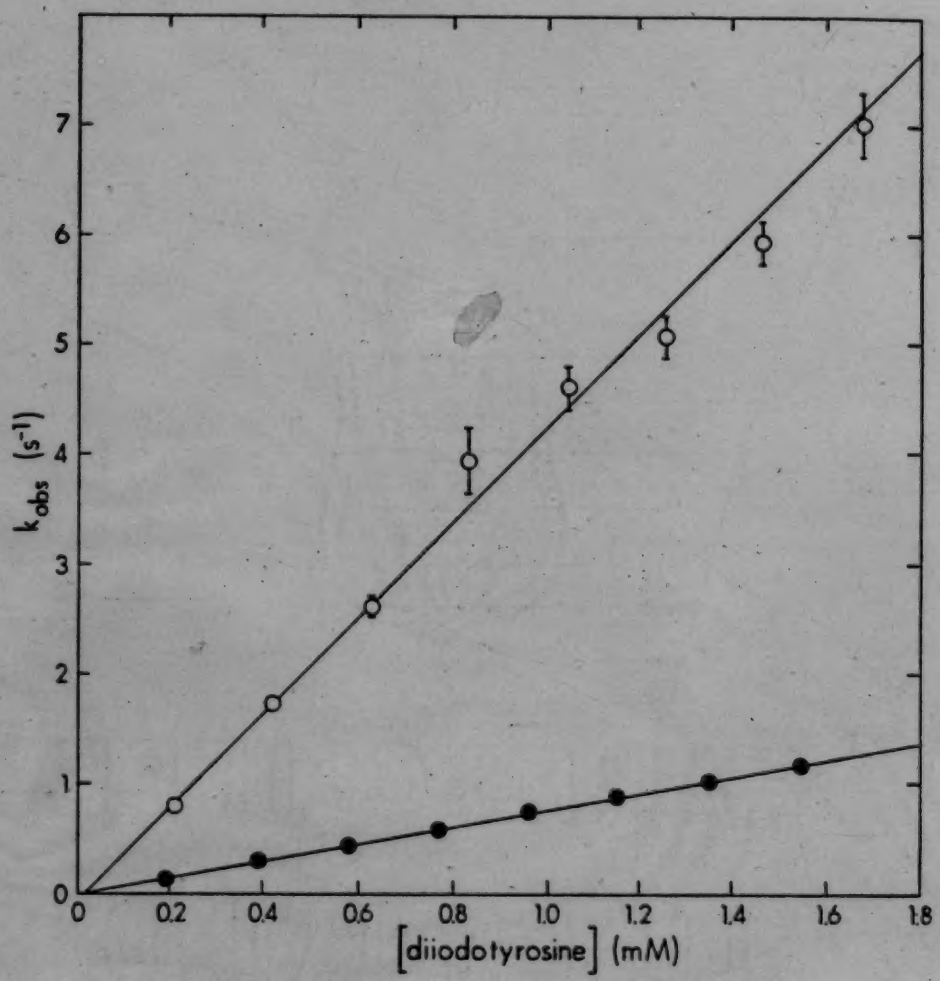


Fig. 3.2 Linear plots of k_{obs} versus [diiodotyrosine] for the reaction of compound II with diiodotyrosine. The slopes correspond to k_{app}, the second order rate constant. The intercepts which are zero within the standard deviation and the slopes are calculated by a weighted linear least squares analysis. ●, pH = 8.35, tris-HNO_3 buffer; O, pH = 6.63, phosphate buffer.

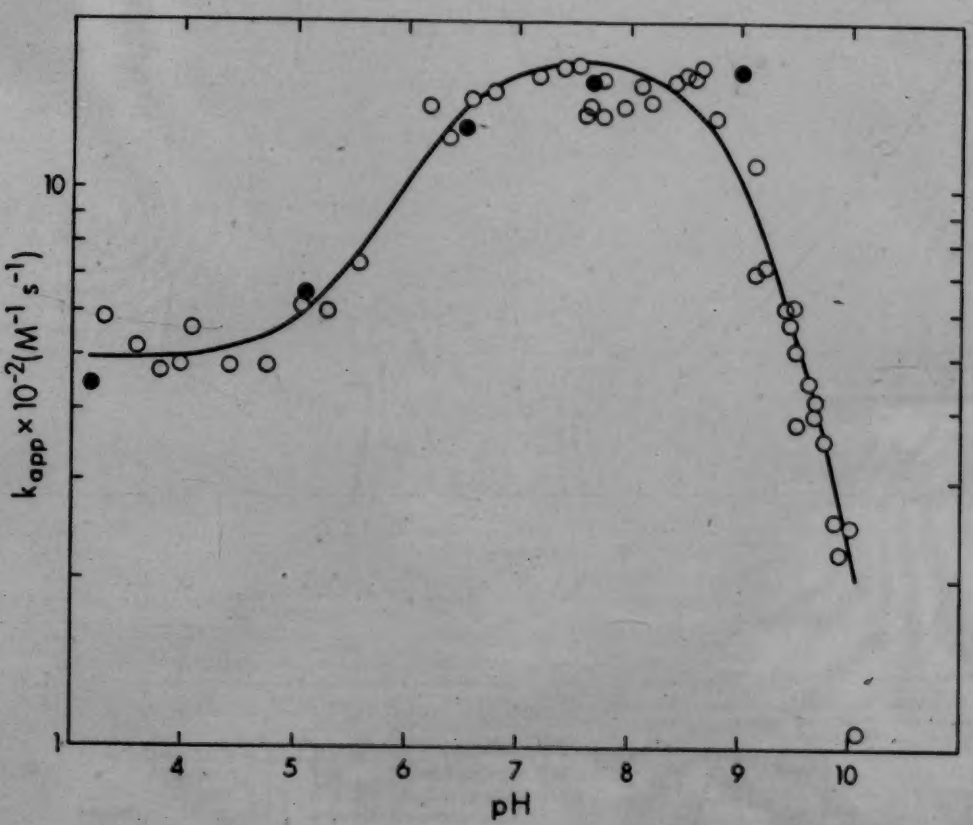


Fig. 3.3 Log (k_{app}) versus pH profile for the reaction of compound II with tyrosine. ●, k_{app} determined from the slopes of plots k_{obs} versus [tyrosine]. O, k_{app} determined by dividing k_{obs} by appropriate [tyrosine]. The best-fit line was found by a weighted nonlinear least squares analysis using Eq. 3.3.

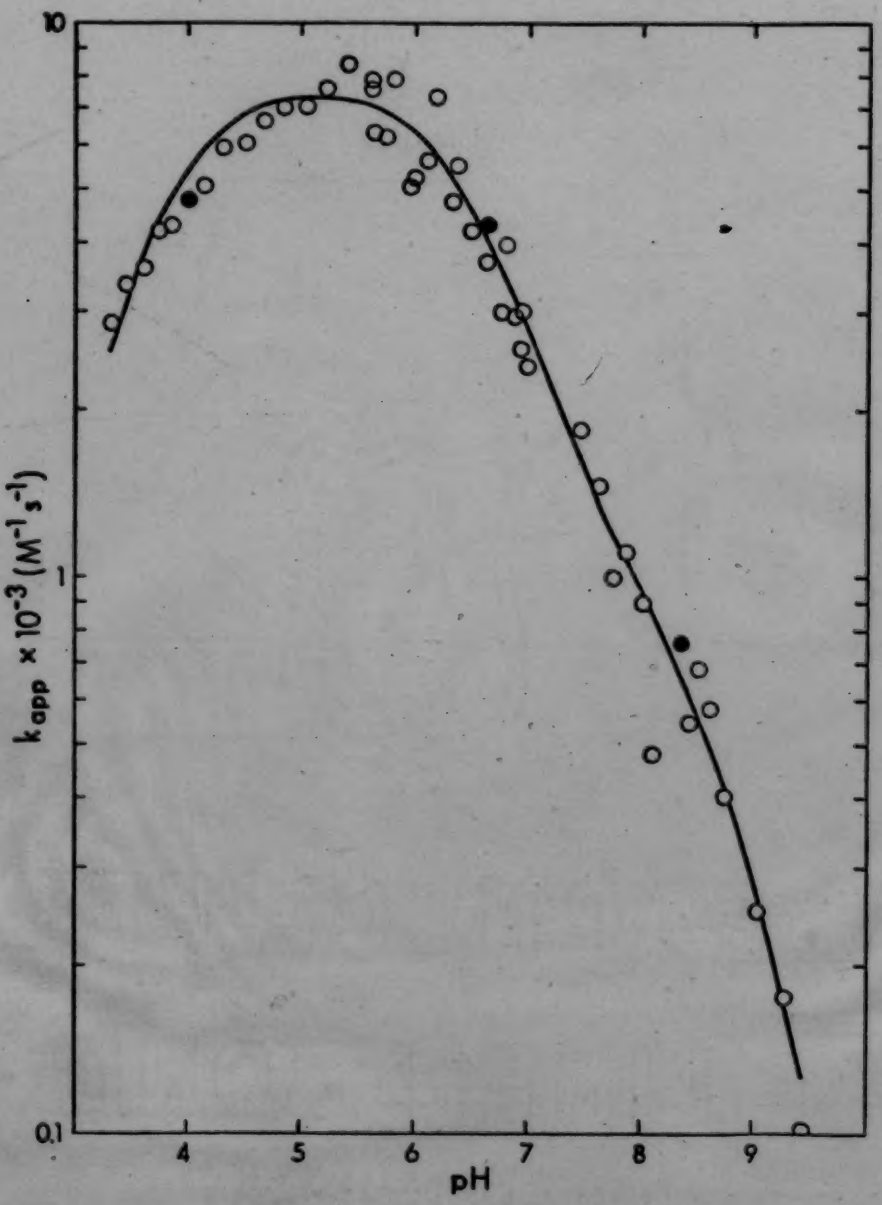


Fig. 3.4 Log (k_{app}) versus pH profile for the reaction of compound II with diiodotyrosine. ●, k_{app} determined from the slopes of plots of k_{obs} versus [diiodotyrosine]. O, k_{app} determined by dividing k_{obs} by appropriate [diiodotyrosine]. The best-fit line was found by a weighted nonlinear least squares analysis using Eq. 3.5.

Table 3.1 Values of k_{app} as a Function of pH for the Reaction of Compound II and Tyrosine

pH	Buffer[a]	$k_{app} \times 10^{-2}$ ($M^{-1}s^{-1}$) [b]	[tyrosine] $\times 10^{3}$ (M)
3.18	CI	4.5 ± 0.2[c]	0.18 - 1.26
3.29	CI	5.9 ± 0.3	0.91
3.57	CI	5.2 ± 0.3	0.91
3.79	CI	4.7 ± 0.2	0.91
3.97	A	4.8 ± 0.1	0.91
4.08	CI	5.6 ± 0.4	0.54
4.42	CI	4.8 ± 0.3	0.91
4.75	CI	4.8 ± 0.3	0.91
5.06	CI	6.2 ± 0.3	0.91
5.10	P	6.5 ± 0.3[c]	0.18 - 1.45
5.28	CI	6.0 ± 0.2	0.91
5.57	CI	7.4 ± 0.6	0.91
6.20	P	14 ± 2	1.22
6.37	P	12 ± 1	0.92
6.53	P	13.0 ± 0.8[c]	0.20 - 1.57
6.58	P	14.5 ± 0.7	1.22
6.77	P	15 ± 2	1.22
7.19	P	16.1 ± 0.4	1.22
7.41	P	16.6 ± 0.7	1.22
7.53	P	16.8 ± 0.3	1.22
7.61	PH	13.7 ± 0.5	1.10
7.65	T	14.2 ± 0.5	1.11
7.68	P	15.7 ± 0.4[c]	0.20 - 1.56
7.76	T	13.6 ± 0.5	1.11
7.76	PH	15.8 ± 0.5	1.10
7.95	T	14.2 ± 0.8	1.11
8.10	PH	15.4 ± 0.9	1.10
8.20	T	14.4 ± 0.7	1.11
8.41	T	15.7 ± 0.7	1.11
8.51	PH	16.2 ± 0.5	1.10
8.59	T	16.0 ± 0.5	1.11
8.65	T	16.7 ± 0.5	1.11
8.78	PH	13.5 ± 0.2	1.11
9.01	T	16.3 ± 0.4[c]	0.21 - 1.66
9.13	PH	11.2 ± 0.4	1.10
9.15	PH	7.1 ± 0.7	1.44
9.23	PH	7.3 ± 0.8	1.24
9.41	PH	6.2 ± 0.2	1.03
9.45	C	5.8 ± 0.1	1.34
9.50	PH	6.2 ± 0.2	1.10
9.50	C	5.2 ± 0.2	1.15

Table 3.1, Continued

pH	Buffer[a]	$k_{app} \times 10^{-2}$ ($M^{-1}s^{-1}$)[b]	[tyrosine] $\times 10^{3}$ (M)
9.52	PH	3.8 ± 0.1	0.86
9.62	C	4.5 ± 0.8	0.96
9.67	C	4.0 ± 0.1	0.77
9.68	PH	4.2 ± 0.2	1.10
9.76	C	3.6 ± 0.1	0.58
9.87	C	3 ± 1	0.89
9.91	PH	2 ± 1	0.62
10.00	PH	2.5 ± 0.2	1.10
10.07	C	1.07 ± 0.06	0.89

[a]Buffer Key: A, acetate; CI, citrate; P, phosphate; PH, phosphate-NaOH; T, tris-HNO_3; C, carbonate.

[b]k_{app} determined by dividing the pseudo-first order rate constant by the appropriate tyrosine concentration, unless otherwise indicated. Error is the standard deviation of the mean value of k_{app}.

[c]k_{app} determined from the slope of the plot of k_{obs} versus [tyrosine]. Error is the standard deviation calculated by the least squares analysis.

Table 3.2 Values of k_{app} as a Function of pH for the Reaction of Compound II and Diiodotyrosine

pH	Buffer[a]	$k_{app} \times 10^{-2}$ ($M^{-1}s^{-1}$)[b]	[diiodotyrosine] $\times 10^{3}$ (M)
3.31	CI	29 ± 3	0.37
3.45	CI	34 ± 1	0.37
3.62	CI	36 ± 2	0.37
3.74	CI	42 ± 1	0.37
3.85	CI	43 ± 3	0.37
4.00	A	48 ± 2[c]	0.19 - 1.35
4.13	CI	51 ± 2	0.37
4.30	CI	60 ± 3	0.37
4.50	CI	61 ± 3	0.37
4.67	CI	67 ± 2	0.37
4.84	CI	70 ± 2	0.37
5.03	CI	71 ± 6	0.37
5.21	CI	76 ± 6	0.37
5.39	CI	84 ± 6	0.37
5.61	CI	79 ± 3	0.37
5.61	CI	76 ± 3	0.37
5.64	P	63 ± 3	1.26
5.74	P	62.3 ± 0.8	1.26
5.81	CI	79 ± 2	0.37
5.96	P	50 ± 7	1.26
5.99	P	52 ± 3	1.31
6.11	P	56 ± 2	1.31
6.18	P	73.3 ± 0.8	1.26
6.32	P	48 ± 2	1.26
6.37	P	55 ± 4	1.31
6.49	P	42.1 ± 0.6	1.26
6.62	P	37 ± 2	1.26
6.63	P	43 ± 1[c]	0.22 - 1.79
6.76	P	30 ± 1	1.26
6.80	P	40 ± 2	1.31
6.86	P	30 ± 2	1.26
6.92	P	25.8 ± 0.9	1.26
6.93	P	30 ± 2	1.31
6.98	P	24 ± 2	1.26
7.47	T	19 ± 2	1.31
7.63	T	14.7 ± 0.8	1.31
7.77	C	10.0 ± 0.5	1.51
7.88	T	11.2 ± 0.3	1.31
8.02	C	9.0 ± 0.4	1.51

Table 3.2, Continued

pH	Buffer[a]	$k_{app} \times 10^{-2}$ ($M^{-1}s^{-1}$) [b]	[diiodotyrosine] $\times 10^{3}$ (M)
8.09	T	4.8 ± 0.2	1.31
8.35	T	7.62 ± 0.09 [c]	0.21 - 1.65
8.43	C	5.5 ± 0.6	1.51
8.51	T	6.8 ± 0.4	1.31
8.62	T	5.8 ± 0.5	1.31
8.74	C	4.0 ± 0.2	1.51
9.04	C	2.50 ± 0.07	1.51
9.28	C	1.75 ± 0.04	1.51
9.43	C	1.01 ± 0.06	1.51

[a]Buffer Key: A, acetate; CI, citrate; P, phosphate; T, tris-HNO_3; C, carbonate.

[b]k_{app} determined by dividing the pseudo-first order rate constant by the appropriate diiodotyrosine concentration, unless otherwise indicated. Error is the standard deviation of the mean value of k_{app}.

[c]k_{app} determined from the slope of the plot of k_{obs} versus [diiodotyrosine]. Error is the standard deviation calculated by the least squares analysis.

substrate concentration.

When the solutions are prepared at pH 4.16, using only citrate buffer of ionic strength 0.11 (instead of adjusting the ionic strength with 0.10 M potassium nitrate), the rates of reaction for either tyrosine or diiodotyrosine with compound II remain unchanged. In the absence of nitrate, the k_{app} values for the tyrosine-compound II or diiodotyrosine-compound II reactions respectively are $(5.4 \pm 0.7) \times 10^2 M^{-1}s^{-1}$ and $(5.6 \pm 0.4) \times 10^3 M^{-1}s^{-1}$. When plotted, each point falls very near the best-fit line of the appropriate graph. Hence, if nitrate binding to compound II does occur, the rate of reaction with either tyrosine or diiodotyrosine is not influenced.

3.5 Discussion

For the compound II reaction with tyrosine several alternatives must be considered when analyzing the profile of log (k_{app}) versus pH. Two major observations are noted. Firstly, as shown in Fig. 3.3, the reaction rate increases with increasing pH between pH 5 and 7 and, secondly, a dramatic decrease in k_{app} is noted above pH 8. One mechanism which may account for these observations is given by the following scheme.

[3.2]

$$\begin{array}{ccc} EH + SH & \xrightarrow{k_1} & \text{products} \\ K_E \uparrow\downarrow & & \\ E + SH & \xrightarrow{k_2} & \text{products} \\ K_S \uparrow\downarrow & & \\ S & & \end{array}$$

E, EH, S and SH are the basic and acidic forms of the enzyme and substrate respectively with ionization constants K_E and K_S; whereas, k_1 and k_2 are the second order rate constants for the reactions involving the protonated and deprotonated forms of the enzyme. In the pH range of study, only the amino group dissociation of tyrosine is considered since the acidic form of the carboxyl group and the basic form of the phenolic group are present in small concentrations; hence, there is less likelihood of these forms exerting an influence on changes in the reaction rate (12). Using scheme 3.2, Eq. 3.3 is derived as shown in Section 3.6 and the best-fit parameter values and standard deviations are shown in Table 3.3.

$$[3.3] \qquad k_{app} = \frac{k_1 + \frac{k_2 K_E}{[H^+]}}{\left(\frac{K_E}{[H^+]} + 1\right)\left(\frac{K_S}{[H^+]} + 1\right)}$$

pK_S is in good agreement with the literature value of 9.11 for the dissociation of the tyrosine amino group (13) and the theoretical curve drawn in Fig. 3.3 gives a reasonable fit to the data.

If scheme 3.2 does indeed represent the mechanism for the oxidation of tyrosine by compound II, then in the region where the reaction rate reaches a maximum (between pH 7 and 8) the basic form of the enzyme and the acid form of the substrate are both present in largest concentrations. Depro-

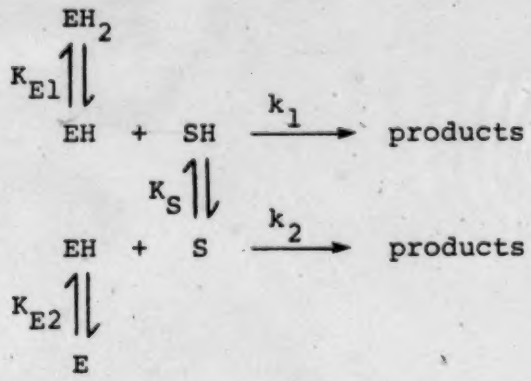
Table 3.3 Kinetic Parameters for the log (k_{app}) versus pH Profile for the Reaction of Compound II with Tyrosine

k_1, $M^{-1}s^{-1}$	$(5.0 \pm 0.3) \times 10^2$
k_2, $M^{-1}s^{-1}$	$(2 \pm 1) \times 10^3$
K_E, M	$(7 \pm 2) \times 10^{-7}$
pK_E	6.2 ± 0.1
K_S, M	$(6.9 \pm 0.6) \times 10^{-10}$
pK_S	9.16 ± 0.04

tonation of the enzyme group with a pK_a value of 6.2 slightly enhances the rate of reaction; a similar effect was observed at pH 5.7 for the compound II reaction with *p*-cresol (6). A second ionization with a pK_a of 9.16 corresponding to the amino group of tyrosine, results in a large decrease in k_{app}. However, in spite of the good correlation between the kinetic determination of the amino pK_a of tyrosine and that given in the literature, the possibility that the change in rate at pH > 8, as illustrated in Fig. 3.3, results from the deprotonation of a second enzyme acid group cannot be rejected. Previous studies of the reactions of compound II with various substrates (ie. ferrocyanide (1-3), *p*-aminobenzoic acid (4,5) and *p*-cresol (6)) have shown the common feature of an enzymatic pK_a of approximately 8.6. It is conceivable that the calculated pK_a value of 9.16 for the tyrosine-compound II reaction corresponds to the same enzyme ionization. With the present available knowledge, however, one cannot positively ascertain to which ionization (substrate or enzyme) the pK_a value of 9.16 should be assigned.

For the reaction of 3,5-diiodo-L-tyrosine with compound II, the assignment of the pK_a values seems less ambiguous. As shown in Fig. 3.4, the reaction rate is at a maximum at pH 5.2 and decreases with increasing acidity or basicity. The following mechanism may describe such behaviour.

[3.4]



EH_2, EH and E are the most, intermediate and least protonated forms of the enzyme; whereas, SH and S correspond to the acidic and basic forms of the substrate phenolic group. k_1 and k_2 are the second order rate constants; K_S, K_{E1} and K_{E2} are the ionization constants for the substrate group, and two enzyme moieties respectively. The ionizations for the carboxyl and amino groups of diiodotyrosine lie outside the pH range of study and hence may not make significant contributions to changes in reaction rate. Eq. 3.5, derived from this scheme (see Section 3.6), has five parameters.

$$[3.5] \qquad k_{app} = \frac{k_1 + \frac{k_2 K_S}{[H^+]}}{\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right)\left(1 + \frac{K_S}{[H^+]}\right)}$$

The numerical values of these parameters and their standard deviations which were determined by a nonlinear least squares method are summarized in Table 3.4. The best-fit line coincides with the data points and the value of pK_S corresponds well with the spectrophotometrically evaluated

Table 3.4 Kinetic Parameters for the log (k_{app}) versus pH Profile for the Reaction of Compound II with Diiodotyrosine

k_1, $M^{-1}s^{-1}$	$(7.7 \pm 0.4) \times 10^3$
k_2, $M^{-1}s^{-1}$	$(1.0 \pm 0.4) \times 10^3$
K_{E1}, M	$(2.4 \pm 0.4) \times 10^{-4}$
pK_{E1}	3.6 ± 0.1
K_{E2}, M	$(2.5 \pm 0.6) \times 10^{-9}$
pK_{E2}	8.6 ± 0.1
K_S, M	$(2.7 \pm 0.5) \times 10^{-7}$
pK_S	6.6 ± 0.1

pK_a (6.37) for the phenolic dissociation of diiodotyrosine (Chapter II). A second mechanism involving the reaction, $EH_2 + S \xrightarrow{k_1}$ products, cannot be discounted on the basis of kinetic evidence alone.

It is not unexpected that the reaction rate of diiodotyrosine with compound II is greatest with protonation of the phenolic group. A previous study involving compound II and *p*-cresol revealed that removal of the phenolic proton results in a large decrease in k_{app} (6); hence, the reaction of the fully protonated enzyme with the phenolate form of diiodotyrosine seems less plausible. The two enzyme acid groups, at pH 3.6 and pH 8.6 for the diiodotyrosine reaction have opposite effects on the reaction rate. The group with pK_a 3.6 increases the rate slightly when in its basic form. The second enzyme dissociation occurring at pH 8.6 has been observed for compound II reactions with other substrates (1-6).

The mechanism of oxidation of substrates by compound II is not entirely clear and requires discussion. First of all, there appears, for the compound II reactions with *p*-cresol (6), tyrosine and diiodotyrosine, an enzyme acid group of pK_a 5.7, 6.2 and 3.6 respectively which slightly affects the reaction rate. This particular group which may or may not be identical in all three cases possibly functions as a secondary proton acceptor for the phenolic proton thereby enhancing the reaction rate to a small degree. This postulation concurs with a high pressure stopped-flow experiment

involving the oxidations of tyrosine, monoiodotyrosine, diiodotyrosine and *p*-aminobenzoic acid by compound II (14). The negative activation volumes for these reactions at pH 7 may be interpreted as proton transfer from a neutrally charged substrate group to a neutral basic group (perhaps a histidyl residue) on the enzyme. It had previously been suggested for the *p*-cresol - compound II reaction that the enzyme group of pK_a 5.7 does not lie immediately in the reaction site and is therefore only indirectly involved in base catalysis (6).

It is possible that an overall charge of zero on the substrate (*p*-cresol, tyrosine or diiodotyrosine) is preferable for compound II reactions. It may be this factor which limits the reactivity of the phenolate forms of *p*-cresol and diiodotyrosine. This may also explain the decrease in k_{app} with deprotonation of the amino moiety of tyrosine. One should not otherwise expect the ionization of this group to influence the rate since it seems unlikely that electron (or hydrogen atom) transfer would occur at an aliphatic side chain. The opposite effect was noted for the oxidation of tyrosine by compound I where the rate increases with deprotonation of the amino group (Chapter II, (15)). The combined electrostatic and conformational effects which possibly differ for compounds I and II may be responsible (16).

The most significant observation for the compound II reactions is the enzyme acid group of $pK_a \sim 8.6$ which has a large effect on the compound II reactions with *p*-cresol (6), diiodotyrosine, ferrocyanide (1-3), *p*-aminobenzoic acid (4,5) and possibly tyrosine. This group is thought to be a

distal group (perhaps a lysine residue) which, in its protonated form, promotes electron transfer from the substrate to the ferryl ion by partial proton transfer to the histidyl residue occupying the fifth coordination position of the heme (17). This explanation may account for the major difference between the compound I and the compound II reactions with substrates such as *p*-cresol, tyrosine and diiodotyrosine. The compound I reactions are base-catalyzed by an enzyme group of $pK_a \sim 5$; whereas, the compound II reactions are acid-catalyzed by an enzyme group of pK_a 8.6.

As illustrated in Fig. 1.4, a second enzyme dissociation of $pK_a \sim 0$ has a large effect on the oxidation rates of ferrocyanide, *p*-aminobenzoic acid, iodide, bisulphite and nitrous acid by compound II. This has been attributed to protonation of the imidazole ligand in the fifth coordination position by hydronium ion (17). Specific acid catalysis is not observed for the reactions of compound II with *p*-cresol and the tyrosines.

3.6 Derivations of Eqs. 3.3 and 3.5

Derivation of Eq. 3.3:

$$v = k_{app} [E]_{total} [S]_{total} = k_1[EH][SH] + k_2[E][SH]$$

$$[E]_{total} = [E] + [EH]$$

$$[S]_{total} = [S] + [SH]$$

$$k_{app} = \frac{k_1[EH][SH] + k_2[E][SH]}{([E]+[EH])([S]+[SH])}$$

$$k_{app} = \frac{k_1 + \frac{k_2 K_E}{[H^+]}}{\left(\frac{K_E}{[H^+]} + 1\right)\left(\frac{K_S}{[H^+]} + 1\right)}$$

Derivation of Eq. 3.5:

$$v = k_{app}\,[E]_{total}[S]_{total} = k_1[EH][SH] + k_2[EH][S]$$

$$[E]_{total} = [E] + [EH] + [EH_2]$$

$$[S]_{total} = [S] + [SH]$$

$$k_{app} = \frac{k_1[EH][SH] + k_2[EH][S]}{([E]+[EH]+[EH_2])([S]+[SH])}$$

$$k_{app} = \frac{k_1 + \frac{k_2 K_S}{[H^+]}}{\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right)\left(1 + \frac{K_S}{[H^+]}\right)}$$

3.7 Acknowledgement

A preliminary study of the compound II reaction with tyrosine was carried out by Dr. H.C Malhotra.

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CHAPTER IV.

HORSERADISH PEROXIDASE-CATALYZED IODINATION OF TYROSINE

4.1 Summary

The horseradish peroxidase-catalyzed reaction of iodide with tyrosine was studied as a function of pH. A prominent change in the reaction kinetics was observed at pH ~ 5, corresponding to the ionization of an acid group of horseradish peroxidase compound I. Several mechanisms for the reaction under acid conditions were eliminated by the comparison of the measured rate constants with calculated theoretical values. A mechanism involving an "$I^{\oplus}$" species reacting with tyrosine to yield monoiodotyrosine may be operative at low pH. The presence of a free radical species was not detected by an epr experiment. In basic solution, the coupling of tyrosyl radicals to form bityrosine is likely the favoured reaction. No enzyme-iodine complex was observed when molecular iodine was added to the ferric enzyme.

4.2 Introduction

Because of the physiological significance of tyrosine iodination, several studies have been devoted to the peroxidase-catalysis of this particular reaction (1-19). Under basic conditions molecular iodine reacts spontaneously with the phenolate form of tyrosine; however, at low pH the reaction rate is slower. In acidic, basic, or neutral solutions, iodide ion and tyrosine are unreactive, unless peroxidase is present as a catalyst.

One of the major questions concerning the formation of monoiodotyrosine from iodide and tyrosine is whether the peroxidase-catalyzed reaction proceeds via a free radical mechanism (ie. combination of atomic iodine with a tyrosyl radical) or via attack of an "$I^{\oplus}$" species on tyrosine (20). Another point of contention is whether the iodination of tyrosine takes place in solution or within the structure of the enzyme (3,6-9,12,13,15,17,19). The mechanistic details of the horseradish peroxidase-catalyzed reaction of iodide with tyrosine are outlined in this chapter.

4.3 Experimental Procedure

4.3.1 Materials

The horseradish peroxidase (EC 1.11.1.7 donor-H_2O_2 oxidoreductase, lot 1399140) was purchased as an ammonium sulphate precipitate from Boehringer-Mannheim Corp. After dialysis in quintuply-distilled water (21) a purity number of 3.25 was determined from the ratio of absorbances at 403 and 280 nm. The concentration of the enzyme solution was established by spectrophotometric measurement at 403 nm where the molar absorptivity coefficient is $1.02 \times 10^5\ M^{-1}\ cm^{-1}$ (22).

Reagent grade potassium iodide and L-(-)-tyrosine were obtained from Shawinigan and Eastman Kodak respectively. Solutions were prepared daily and their concentrations were determined by weight. The standardization procedure for hydrogen peroxide has been reported elsewhere (23).

4.3.2 Apparatus and Methods

Absorbance measurements were conducted on a Cary 219 spectrophotometer and pH was measured with a Fisher model 420 pH meter in combination with a Fisher electrode. Experiments where the monitoring of fast reactions was required were carried out on a Union-Giken stopped-flow spectrophotometer model RA-601 equipped with a thermostatted 1 cm cell.

The initial rates of reaction were determined. The rate of appearance of monoiodotyrosine was monitored at 290 nm using $\Delta\varepsilon$ (2.34×10^3 M^{-1} cm^{-1} (10)), the difference in the molar absorptivities of monoiodotyrosine and tyrosine. Triiodide ion formation or consumption was followed at either 290 nm ($\varepsilon = 4.00 \times 10^4$ M^{-1} cm^{-1} (24)) or 353 nm ($\varepsilon = 2.55 \times 10^4$ M^{-1} cm^{-1} (23)). The change in concentration of molecular iodine with time was determined at 460 nm ($\varepsilon = 7.4 \times 10^2$ M^{-1} cm^{-1} (24)). The initial rates of molecular iodine or triiodide ion formation were determined by taking the tangent to the curve at zero time (Fig. 4.1). To estimate the rate of initial production of monoiodotyrosine the tangent was drawn to the curve at the point where monoiodotyrosine formation was first observed (Fig. 4.2). At low pH the error associated with this method is small since essentially all of the triiodide is converted to molecular iodine before monoiodotyrosine formation takes place and there is hence little overlap in the absorbances due to triiodide ion and monoiodotyrosine. At higher pH values, however, the initial

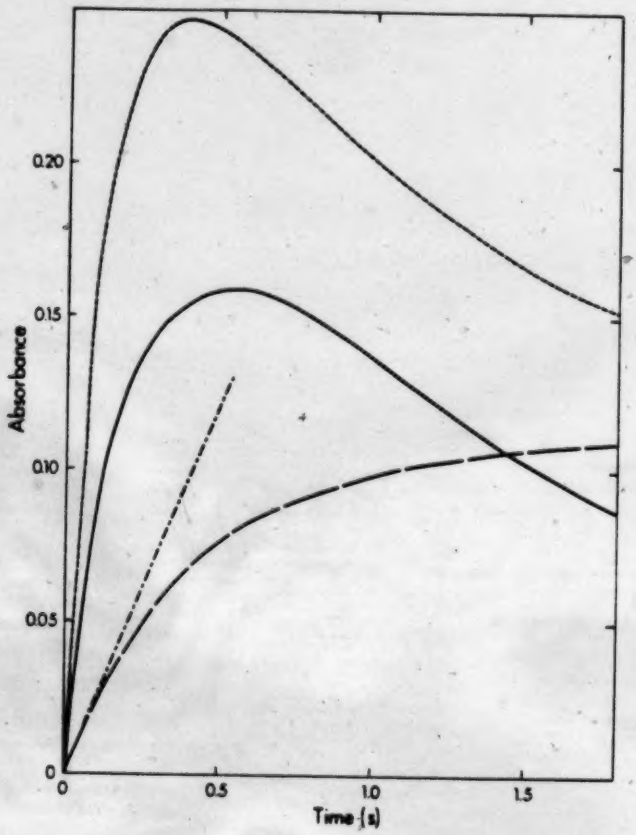

Fig. 4.1 Formation and disappearance of triiodide ion and molecular iodine during the horseradish peroxidase-catalyzed iodination of tyrosine: (---) triiodide ion measured at 290 nm; (—) triiodide ion measured at 353 nm; (— — —) molecular iodine measured at 460 nm. Solution is 0.01 ionic strength in citrate buffer of pH 3.68 and 0.10 ionic strength in potassium sulphate. Initial concentrations are: $[KI] = [\text{tyrosine}] = 3.24 \times 10^{-4}$ M; $[H_2O_2] = 1.11 \times 10^{-3}$ M; $[HRP] = 1.73 \times 10^{-6}$ M. Stopped-flow procedure enabled rapid mixing of solutions. (— - —) Initial rate determined from tangent to the curve.

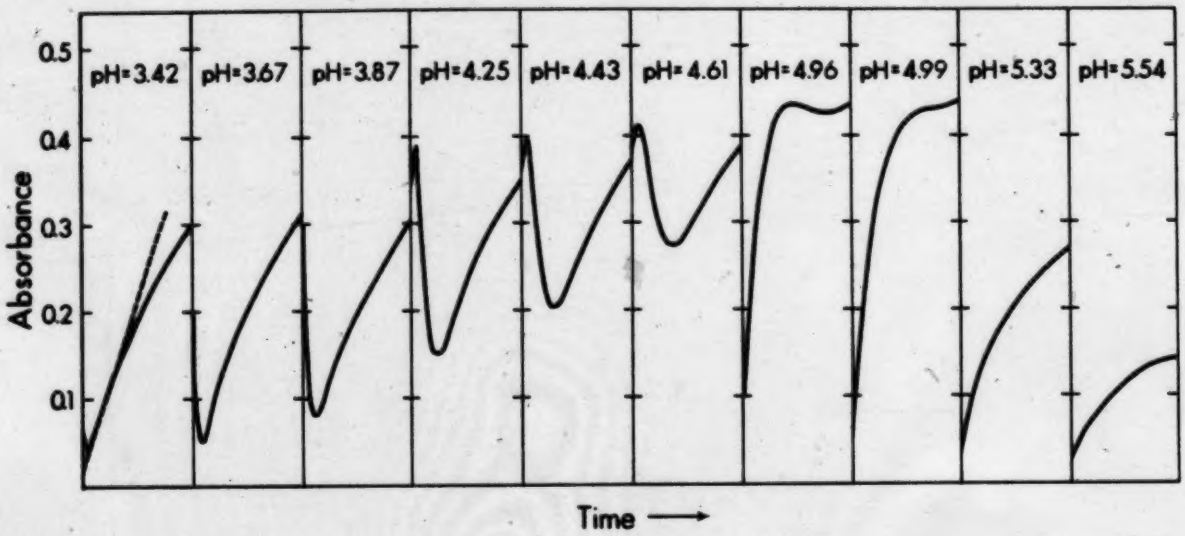

Fig. 4.2 pH Dependence of the horseradish peroxidase-catalyzed tyrosine iodination. Reaction monitored at 290 nm. Each reaction studied over a period of 60 s. All solutions are 0.01 ionic strength in citrate buffer and 0.10 ionic strength in potassium sulphate. Initial concentrations are: [KI] = [tyrosine] = 3.24×10^{-4} M; $[H_2O_2] = 1.11 \times 10^{-3}$ M; [HRP] = 4.33×10^{-7} M. The reaction was initiated by addition of enzyme. (---) Initial rate determined from tangent to the curve.

rate of monoiodotyrosine formation cannot be measured with confidence.

All solutions were maintained at 25°C and at a constant total ionic strength (0.10 due to potassium sulphate and 0.01 due to buffer). For the stopped-flow experiments, citrate buffer of pH 3.68 was used. One storage cell contained a solution of 3.24×10^{-4} M potassium iodide and 1.73×10^{-6} M horseradish peroxidase; whereas, a solution of 1.11×10^{-3} M hydrogen peroxide (and 3.24×10^{-4} M tyrosine, when applicable) was stored in the second cell. Experimental conditions for other steady state reactions at pH 3.7 are described in Table 4.1. In the study of the pH dependence of tyrosine iodination, citrate buffer was employed for all solutions, except for the experiment at pH 8.37 where tris-HNO_3 buffer was used. The initial concentrations of reactants in the 2 ml sample were 3.24×10^{-4} M potassium iodide, 3.24×10^{-4} M tyrosine and 1.11×10^{-3} M hydrogen peroxide. The reactions were initiated by the addition of 4.33×10^{-7} M horseradish peroxidase.

For the qualitative test on the reaction products of the horseradish peroxidase-catalyzed oxidation of iodide, the 3 ml sample contained 9.25×10^{-3} M hydrogen peroxide, 1.08×10^{-3} M potassium iodide and 5.16×10^{-7} M enzyme. After incubation for 10 min, silver nitrate was added to the mixture. The precipitate which formed was filtered, washed and dissolved in 0.5 N HCl. Carbon tetrachloride and potassium thiocyanate were then added and the mixture was shaken (25).

Table 4.1 Initial Rates of Tyrosine Iodination at Low pH

Conditions[a]	Wavelength (nm)	Species monitored[b]	Initial Reaction rate ($M\,s^{-1}$)
$[I_2] = 8.1 \times 10^{-5}$ M[c]	290	MIT	1.0×10^{-7}
	460	I_2	5.2×10^{-8}
$[H_2O_2] = 2.78 \times 10^{-4}$ M $[I^-] = 1.62 \times 10^{-4}$ M [HRP] = 3.43×10^{-7} M[c]	290	MIT	5.4×10^{-7}
	460	I_2	2.8×10^{-7}
$[H_2O_2] = 2.78 \times 10^{-4}$ M $[I_2] = 8.1 \times 10^{-5}$ M [HRP] = 3.43×10^{-7} M[c]	290	MIT	5.5×10^{-7}
	460	I_2	2.0×10^{-7}

[a]All solutions contained 0.01 ionic strength citrate buffer of pH 3.7, 0.10 ionic strength potassium sulphate and 4.54×10^{-5} M tyrosine.

[b]MIT corresponds to monoiodotyrosine formation and I_2 corresponds to the disappearance of molecular iodine.

[c]Reaction initiator.

To determine whether or not molecular iodine binds to the ferric enzyme, 1.22×10^{-3} M iodine was added to 7.62×10^{-6} M horseradish peroxidase. The sample was 0.10 ionic strength in potassium sulphate and 0.01 ionic strength in citrate buffer of pH 3.7. The reference cuvette contained buffer, electrolyte and iodine, but no enzyme.

A Bruker ER400 Consul with a Varian V3601-12 magnet and VFR-2503 field regulator was employed for the epr experiments. Scans were carried out at g ~ 2 for 75 G in either direction. The aqueous samples were contained in flat quartz cells. The reactant concentrations were equivalent to those used in the pH study; the solution was buffered by citrate at pH 3.68. A special cell equipped with two reservoirs enabled continuous mixing and flow of the solutions through the cell; hence, the spectrum was recorded soon after mixing. The iodide and enzyme were held in one compartment of the cell; whereas, tyrosine and hydrogen peroxide were contained in the other.

4.4 Results

Using the stopped-flow procedure, the formation and/or disappearance of triiodide ion and molecular iodine were monitored at 290 (I_3^-), 353 (I_3^-) and 460 (I_2) nm, as shown in Fig. 4.1. The initial rates of molecular iodine formation at 460 nm were found to be 3.3×10^{-4} M s^{-1} and 3.2×10^{-4} M s^{-1} respectively for the horseradish peroxidase-catalyzed oxidation of iodide in the absence and presence of

tyrosine at pH 3.7. These same reactions are shown over a longer time scale in Fig. 4.3.

Similar experiments to those performed by Bayse and Morrison (10) were carried out and the results are given in Table 4.1.

The pH dependence of the horseradish peroxidase-catalyzed reaction of iodide with tyrosine was followed at 290 nm and the results are demonstrated in Figs. 4.2 and 4.4.

4.5 Discussion

The results of steady state experiments carried out by Bayse and Morrison (10) at low pH are verified in the present study. Tyrosine iodination was followed at three wavelengths. At 460 nm the formation and subsequent disappearance of molecular iodine was observed. The change in concentration of triiodide ion was monitored at 353 and 290 nm. Also at 290 nm, the formation of monoiodotyrosine was traced. In agreement with previously recorded data (10), the following observations were made: (a) the rate of monoiodotyrosine formation measured at 290 nm is twice that of molecular iodine consumption followed at 460 nm; (b) essentially all iodide is converted to iodine before tyrosine iodination occurs; (c) the rate of appearance of monoiodotyrosine is unchanged if one mole of molecular iodine is substituted for two moles of iodide. In addition to these results, new observations were recorded. When the spontaneous reaction of tyrosine with molecular iodine was measured,

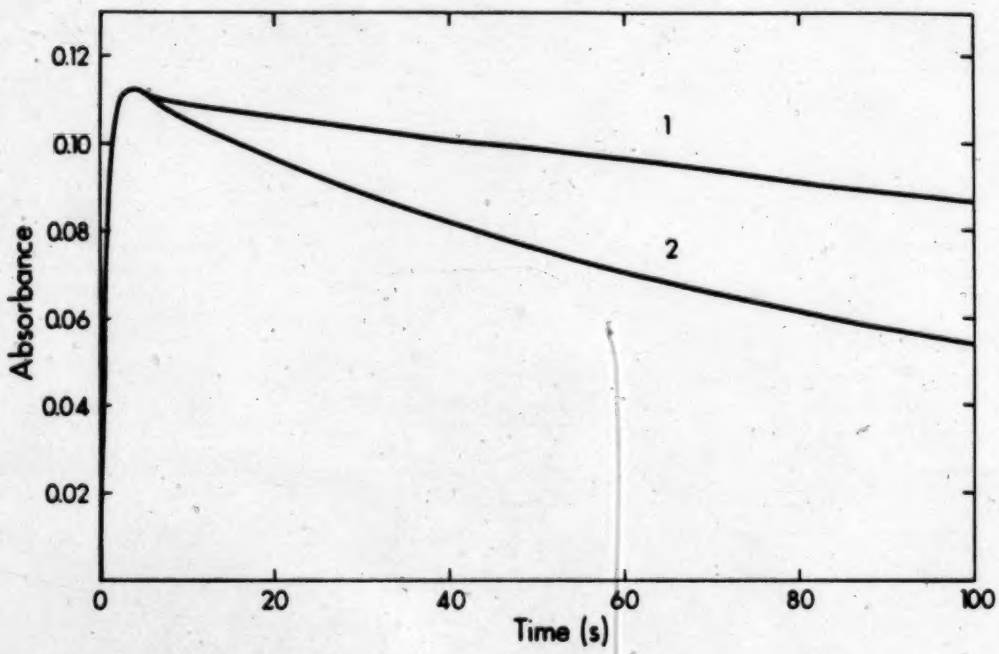

Fig. 4.3 Molecular iodine formation and disappearance at 460 nm: (1) absence of tyrosine; (2) presence of tyrosine. Initial concentrations are given in Fig. 4.1.

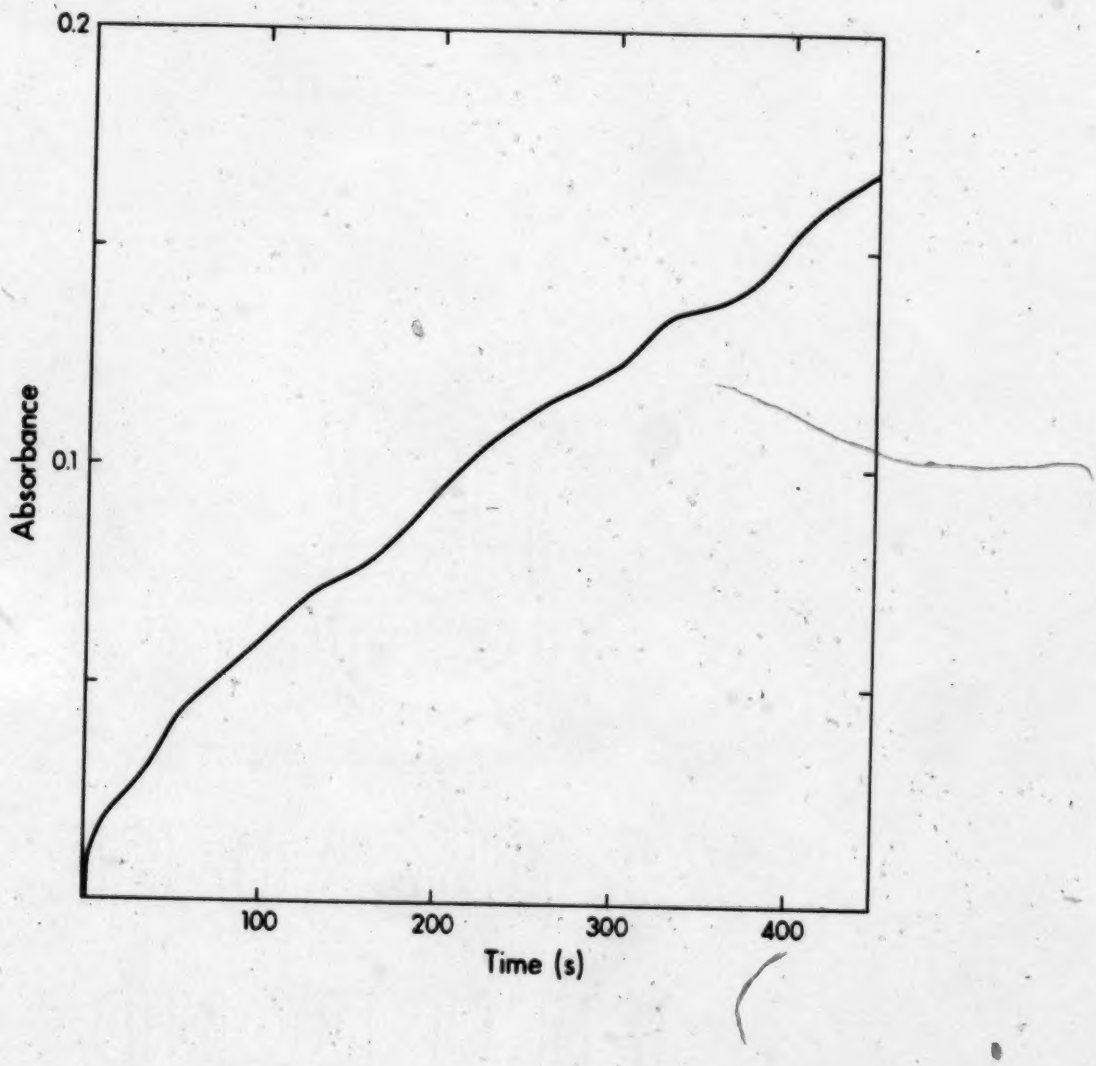

Fig. 4.4 Iodination of tyrosine at pH 8.37. Reaction conditions are the same as those for Fig. 4.2 except that tris-HNO_3 buffer is used.

the rate was at least 5-fold slower than the catalyzed reaction. Using a stopped-flow procedure it was possible to measure and compare the initial rates of molecular iodine formation for the enzyme-catalyzed system in the presence and absence of tyrosine. It was observed that the rate of production of iodine was equal in both cases. All experiments provide evidence that molecular iodine is an intermediate of tyrosine iodination and that peroxidase not only participates by oxidizing iodide to iodine, but, it also catalyzes the further reaction of iodine and tyrosine to yield monoiodotyrosine. This conclusion is not novel and has been discussed previously (10). Both chloro- and lactoperoxidases yield molecular iodine during the reaction of iodide and tyrosine (5,7). It was suggested that iodine is an obligatory intermediate in the case of catalysis by chloroperoxidase (5).

During the study of the horseradish peroxidase-catalyzed oxidation of iodide ion, no iodate was detected. A small amount of yellow precipitate was formed upon the addition of silver nitrate to the incubated reaction mixture of iodide ion, horseradish peroxidase and excess hydrogen peroxide. After dissolving this precipitate in acid, no reaction was observed with the introduction of thiocyanate and carbon tetrachloride. Iodate is expected to form a white precipitate with silver ion; reduction of iodate by thiocyanate yields molecular iodine which is easily identified by its purple colour in carbon tetrachloride. Although it appears

from this study that no iodine species in an oxidation state greater than that of molecular iodine is produced in the horseradish-peroxidase catalyzed oxidation of iodine, it is shown in Fig. 4.2 that molecular iodine does undergo a slow reaction. For the qualitative analysis, the conditions may have been unsuitable for the accumulation of a detectable quantity of iodate. The presence of the yellow precipitate, silver iodide, suggests incomplete oxidation of the iodide. It should be clarified that the described test is also useful for detecting the presence of hypoiodite ion which rapidly disproportionates to iodate and iodide (26,27).

The most striking feature of the study on the pH dependence of tyrosine iodination is the pronounced change in kinetics at pH ~ 5. As shown in Fig. 4.2, the rapid formation of triiodide ion is observed at low pH, followed by its disappearance and the slow appearance of monoiodotyrosine. With increasing basicity, however, triiodide production becomes slower. At pH ~ 5 the effect of diminished triiodide formation on the reaction profile is apparent. The production of monoiodotyrosine continues to be hindered with decreasing acidity. At pH 8.37 another interesting feature is observed; an oscillating reaction is superimposed on the production curve of monoiodotyrosine. It is possible that this oscillating system is the Bray-Liebhafsky reaction which may be summarized by the following alternating reactions (28,29):

[4.1] $$I_2 + 5H_2O_2 \longrightarrow 2IO_3^- + 2H^+ + 4H_2O$$

[4.2] $$2IO_3^- + 5H_2O_2 + 2H^+ \longrightarrow I_2 + 5O_2 + 6H_2O$$

In order to explain the results of the pH study it is necessary to consider the log(rate) versus pH profiles for the oxidations of iodide and tyrosine by both compounds I and II of horseradish peroxidase as illustrated in Fig. 4.5. At very low pH, where the peroxidase-catalyzed iodination of tyrosine is most efficient, iodide oxidation by compound I occurs very rapidly. There is approximately a 1000-fold difference in the rates of iodide and tyrosine oxidations by compound I in the pH region 3 to 4. No compound II is produced during the enzymatic oxidation of iodide. The initial rate of molecular iodine formation may be related to the initial reactant concentrations as shown by Eq. 4.3 (derivation in Section 4.6).

[4.3] $$-\frac{d[I^-]}{dt} = \frac{2\,d[I_2]}{dt} = \frac{k_1[HRP]_o[H_2O_2]}{\left(1 + \frac{k_1[H_2O_2]}{k_2[I^-]}\right)}$$

$[HRP]_o$ is the total concentration of enzyme. Substitution of the initial concentrations of reactants, a k_1 value of $1.5 \times 10^7\ M^{-1}s^{-1}$ (30) and a k_2 value of 1.1×10^6 (Fig. 4.5, (31)), gives an initial rate of $3.1 \times 10^{-4}\ M\,s^{-1}$ for iodine formation which compares favourably with that measured during the steady state analysis ($3.2 \times 10^{-4}\ M\,s^{-1}$). Monoiodotyrosine formation begins only after molecular iodine is

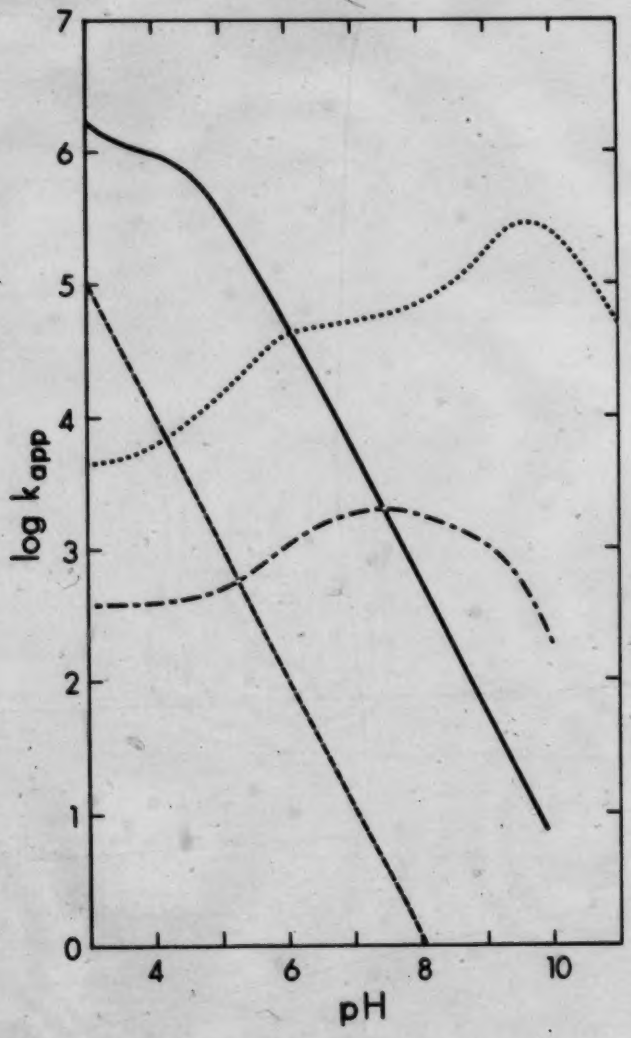

Fig. 4.5 pH Dependencies of oxidations of tyrosine and iodide by both compounds I and II of horseradish peroxidase: (——) iodide oxidation by compound I (30); (---) iodide oxidation by compound II (32); (·····) tyrosine oxidation by compound I (Chapter II, (31)); (— · — ·) tyrosine oxidation by compound II (Chapter III).

produced in almost a stoichiometric yield.

Three mechanisms for the peroxidase-catalyzed iodination of tyrosine must be considered: (a) oxidation of tyrosine to tyrosyl radicals which react with molecular iodine, (b) oxidation of the small amount of iodide remaining in solution to an "$I^{\oplus}$" species which reacts with tyrosine and (c) oxidation of molecular iodine to an "$I^{\oplus}$" species which reacts with tyrosine.

Both compounds I and II of horseradish peroxidase are involved in the steady state cycle of tyrosine oxidation (see Chapters I and II). As shown in Section 4.6, the following equation describing monoiodotyrosine formation may be derived:

$$[4.4] \quad \frac{d[MIT]}{dt} = \frac{2k_1k_3k_4[HRP]_o[H_2O_2][tyr]}{k_1[H_2O_2](k_3+k_4) + k_3k_4[tyr]}$$

where MIT and tyr represent monoiodotyrosine and tyrosine respectively. With substitution of $[H_2O_2] = 1.16 \times 10^{-4}$ M, initial concentrations of tyrosine and enzyme and values for k_3 and k_4 (see Fig. 4.5, Chapters I and II), the rate of monoiodotyrosine formation is calculated to be 1.4×10^{-8} M s^{-1}. This predicted value is lower than the experimental value of 5.4×10^{-7} M s^{-1}. Tyrosine oxidation is therefore too slow to take an active part in monoiodotyrosine production; the mechanism involving tyrosine radicals may be eliminated. Furthermore, an attempt to detect the formation of tyrosyl radicals by an epr experiment failed; no signal was observed

for a solution containing tyrosine, iodide, hydrogen peroxide and enzyme.

Since molecular iodine is produced in almost a stoichiometric yield, the amount of unoxidized iodide present in solution may be estimated by considering the following equilibrium:

$$[4.5] \quad I_2 + H_2O \rightleftharpoons H^+ + I^- + IOH; \; K_{eq} = 6.8 \times 10^{-13} \, M \quad (32)$$

$$[4.6] \quad [I^-] = \sqrt{\frac{6.8 \times 10^{-13} [I_2]}{[H^+]}}$$

Using Eq. 4.6 and assuming a stoichiometric yield of molecular iodine, the concentration of iodide in solution (pH = 3.7) at the time of initial monoiodotyrosine formation is calculated to be 5.3×10^{-7} M. Subsequent substitution of this value, $[H_2O_2] = 1.16 \times 10^{-4}$ M and the initial concentration of enzyme into Eq. 4.3 yields a rate of $2.0 \times 10^{-7} \, M \, s^{-1}$ for iodide disappearance or monoiodotyrosine formation. This value is also somewhat lower than the measured rate of $5.4 \times 10^{-7} \, M \, s^{-1}$; hence, the mechanism involving the oxidation of trace amounts of iodide to an "$I^{\oplus}$" species appears to be in doubt.

The oxidation of molecular iodine to an "$I^{\oplus}$" species which then reacts with tyrosine is an alternative mechanism. Unfortunately no pertinent rate data is available for the peroxidase-catalyzed oxidation of molecular iodine; however, it appears that a slow reaction of iodine takes place in the

absence of tyrosine as shown in Fig. 4.3. The present mechanism is proposed solely by elimination of other plausible schemes. Several authors have suggested the same mechanism (10,17); however, no direct evidence has been presented to prove the involvement of an "$I^{\oplus}$" species.

In neutral or basic solutions, the oxidation of tyrosine by compounds I and II of horseradish peroxidase dominate over iodide oxidation as shown in Fig. 4.5. The combination of tyrosyl radicals to form bityrosine probably takes place before sufficient molecular iodine is produced to react with the oxidized tyrosine. Bityrosine, which also absorbs at 290 nm (33) can be oxidized by the enzyme; however, no pH dependent study has been attempted. If any "$I^{\oplus}$" is produced at high pH, it probably forms hypoiodous acid in solution which in turn may be capable of iodinating bityrosine or tyrosine. It is obvious that under basic conditions the reaction is complicated by many side reactions. Furthermore, many species (tyrosine, monoiodotyrosine, bityrosine, triiodide ion and hypoiodite ion (34)) absorb strongly in the 290 nm region. The onset of the Bray-Liebhafsky reaction gives a more complex system.

A controversy had arisen in the literature over the existence of a horseradish peroxidase - iodine complex. The supposed binding of iodine to the enzyme was first suggested by Björkstén who recorded a new spectrum by adding equimolar quantities of iodide and hydrogen peroxide to the ferric enzyme at pH ~ 6 (8,35). However, this complex was not de-

tected under conditions of high iodide concentration by Roman and Dunford (31). Furthermore, they reported that the molar ratio of compound I reacted to molecular iodine formed is unity; hence no iodine is incorporated into an enzyme-iodine complex in the initial stages of the reaction (31). In the present study, a shift in the spectrum of the ferric enzyme corresponding to that observed by Björkstén was initially noted when iodine was added to the system. However, when the solution in the reference cuvette contained an equimolar quantity of iodine, it was realized that the spectrum of the ferric enzyme had not changed upon addition of iodine. Similarly, no complex formation was observed at pH 6.1 and 8.4. Since water was used as a reference in Björkstén's study (35), the observed spectral changes were due to the production of iodine and the effect of its overlapping absorbance with horseradish peroxidase.

An enzyme-iodine complex was also reported by Nunez and Pommier in a study using labelled iodide and thyroglobulin (3). The horseradish peroxidase was initially incubated with iodide containing trace amounts of ^{131}I. Afterwards, thyroglobulin and iodide labelled with ^{125}I were added to the system. The product, iodinated thyroglobulin, contained more ^{131}I than ^{125}I suggesting that the thyroglobulin reacts with oxidized ^{131}I bound to the enzyme (3). The experimental conditions, however, are suspect since the original concentrations of ^{131}I and ^{125}I are not specified (3). In another paper, Pommier *et al.* (13), give evidence for two

iodide binding sites on horseradish peroxidase by showing that a sigmoidal relationship between molecular iodine formation and iodide concentration exists at pH ~ 7. At pH ~ 5, however, the sigmoidicity was reduced (13). Nunez and Pommier also claim that oxidized thyroglobulin binds to horseradish peroxidase (6). This study must be carefully approached since glucose-glucose oxidase was used as a hydrogen peroxide generating system and the amount of ^{125}I used to label the thyroglobulin was not mentioned.

It has not yet been established whether an oxidized iodide-enzyme complex takes part in the lactoperoxidase-catalyzed iodination of tyrosine (19). The reaction is thought to involve tyrosine binding to the enzyme (7); this conclusion was based on the fact that D and L isomers are iodinated at different rates. A thyroid peroxidase-bound oxidized iodide intermediate has apparently been isolated (17).

The results of studies involving peroxidases other than horseradish peroxidase or protein acceptors instead of free tyrosine may not necessarily be representative of the system described in the present report. On the other hand, the horseradish peroxidase-catalyzed iodination of free tyrosine is several steps removed from providing a clear understanding of the thyroid gland reaction.

4.6 Derivations of Eqs. 4.3 and 4.4

Derivation of Eq. 4.3:

$$HRP + H_2O_2 \xrightarrow{k_1} HRP\text{-}I + H_2O$$

$$HRP\text{-}I + I^- \xrightarrow{k_2} HRP + "I^{\oplus}"$$

$$"I^{\oplus}" + I^- \xrightarrow{fast} I_2 \quad \text{(excess iodide)}$$

$$"I^{\oplus}" + tyr \xrightarrow{fast} MIT \quad \text{(no excess iodide)}$$

$$k_1[HRP][H_2O_2] = k_2[HRP\text{-}I][I^-] \quad \text{(steady state approximation)}$$

$$[HRP\text{-}I] = \frac{k_1[HRP][H_2O_2]}{k_2[I^-]}$$

$$[HRP]_o = [HRP] + [HRP\text{-}I]$$

$$[HRP] = \frac{[HRP]_o}{\left(1 + \frac{k_1[H_2O_2]}{k_2[I^-]}\right)}$$

$$-\frac{d[I^-]}{dt} = \frac{2d[I_2]}{dt} = \frac{d[MIT]}{dt} = k_1[HRP][H_2O_2]$$

$$-\frac{d[I^-]}{dt} = \frac{k_1[HRP]_o[H_2O_2]}{\left(1 + \frac{k_1[H_2O_2]}{k_2[I^-]}\right)}$$

Derivation of Eq. 4.4:

$$HRP + H_2O_2 \xrightarrow{k_1} HRP\text{-}I + H_2O$$

$$HRP\text{-}I + tyr \xrightarrow{k_3} HRP\text{-}II + tyr_{ox}$$

$$HRP\text{-}II + tyr \xrightarrow{k_4} HRP + tyr_{ox}$$

$$2\,tyr_{ox} + I_2 \xrightarrow{fast} 2\,MIT$$

$$k_1[HRP][H_2O_2] = k_3[HRP\text{-}I][tyr] = k_4[HRP\text{-}II][tyr]$$

(steady state approximation)

$$[HRP\text{-}I] = \frac{k_1[HRP][H_2O_2]}{k_3[tyr]}$$

$$[HRP\text{-}II] = \frac{k_1[HRP][H_2O_2]}{k_4[tyr]}$$

$$[HRP]_o = [HRP] + [HRP\text{-}I] + [HRP\text{-}II]$$

$$[HRP]_o = [HRP]\left(\frac{k_1[H_2O_2]}{k_3[tyr]} + \frac{k_1[H_2O_2]}{k_4[tyr]} + 1\right)$$

$$[HRP] = \frac{k_3k_4[tyr][HRP]_o}{k_1k_4[H_2O_2] + k_1k_3[H_2O_2] + k_3k_4[tyr]}$$

$$[HRP\text{-}I] = \frac{k_1k_4[HRP]_o[H_2O_2]}{k_1k_4[H_2O_2] + k_1k_3[H_2O_2] + k_3k_4[tyr]}$$

$$-\frac{d[tyr]}{dt} = \frac{d[MIT]}{dt} = 2k_3[HRP\text{-}I][tyr]$$

$$\frac{d[MIT]}{dt} = \frac{2k_1k_3k_4[HRP]_o[H_2O_2][tyr]}{k_1k_4[H_2O_2] + k_1k_3[H_2O_2] + k_3k_4[tyr]}$$

Abbreviations:

HRP; horseradish peroxidase

HRP-I; horseradish peroxidase compound I

HRP-II; horseradish peroxidase compound II

$[HRP]_o$; initial concentration of horseradish peroxidase

tyr; tyrosine

tyr_{ox}; tyrosyl radical

MIT; monoiodotyrosine

4.7 Acknowledgement

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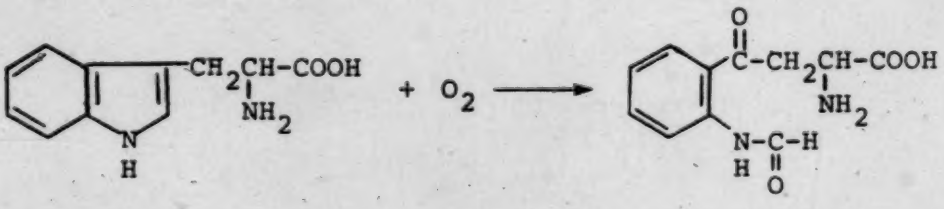
APPENDIX

THE CATALYTIC MECHANISM OF ACTION OF L-TRYPTOPHAN-2,3-DIOXYGENASE

A.1 Introduction

Biological oxidation has been studied in great detail ever since Lavoisier initiated the investigation over 200 years ago. Since that time, the mechanism by which living organisms oxidize organic substances has remained one of the most important and interesting topics in biological science. L-tryptophan-2,3-dioxygenase is one of the many enzymes involved in such oxidation processes.

L-tryptophan-2,3-dioxygenase is a heme-containing enzyme which catalyzes the oxidative ring cleavage of the amino acid, L-tryptophan, to yield N-formylkynurenine;



L-tryptophan N-formylkynurenine

both atoms of molecular oxygen are incorporated in the product. This reaction is the first and one of the rate limiting steps in a metabolic pathway which leads to the biosynthesis of the coenzyme NAD. Tryptophan dioxygenase was first isolated in 1950 (1). The two major sources of the enzyme are rat liver (1) and *Pseudomonas acidovorans* (2).

The bacterial enzyme, which can be obtained in a highly pure form, has a molecular weight of 122,000 (3); whereas, the rat liver enzyme has a molecular weight of 167,000 (4). The enzymes from both sources consist of four subunits and contain two moles of ferriprotoporphyrin IX per mole of protein (4,5). When isolated the heme iron exists in a ferric state with a Soret maximum at 403 nm (6). Reduction with H_2/Pd or sodium dithionite yields the ferrous species; its Soret maximum occurs at 433 nm (6).

Investigations have revealed valuable information about the catalytic mechanism of tryptophan dioxygenase. The involvement of an oxygenated intermediate (7-11) and the allosteric behaviour of the enzyme (12-15) are well established facts; however, controversies over the presence of copper in the enzyme (5,16-25) and the valence state of the heme iron during the various stages of catalysis (26-31) remained unresolved until very recently (32).

A.2 The Oxygenated Intermediate

Probably the most significant step in the elucidation of the reaction mechanism of tryptophan dioxygenase was the discovery in 1967 of an oxygenated intermediate (7). It was observed that addition of the substrate, tryptophan, to either the ferrous or native ferric state of the enzyme under anaerobic conditions resulted in slight shifts of the Soret band (26). This observation indicates that tryptophan binds to the enzyme regardless of its oxidation state.

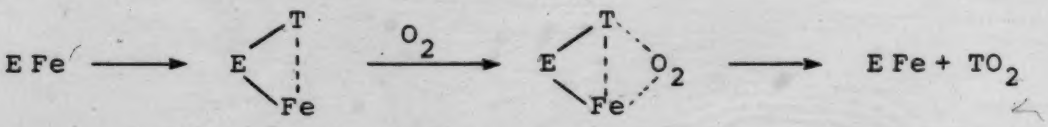
On the other hand, the spectra of both valence forms of the enzyme were essentially unaffected by oxygen in the absence of tryptophan (7). From these observations it appears that the enzyme binds first with the organic substrate and then with oxygen to form a ternary complex (8).

To verify the existence of such a ternary complex, experiments were carried out at 5°C to slow down the rate of reaction (8). When oxygen was bubbled through a solution containing *ferrous* enzyme and tryptophan, a new spectral species immediately appeared. The absorption maxima of this species were observed at 418, 545 and 580 nm which compare favourably with the α, β and γ bands of oxygenated heme proteins such as hemoglobin, myoglobin and peroxidase (11). In contrast to these results, when oxygen was bubbled through a system containing *ferric* enzyme and tryptophan, no spectral changes were observed (7). If oxygen were added to the ferrous enzyme in the absence of tryptophan, the spectrum changed very slowly to that of the ferric enzyme (7). The formation of this new species therefore seems to depend on the simultaneous presence of ferrous heme, tryptophan and oxygen (7-11).

Additional studies were carried out to establish whether or not this ternary complex of tryptophan dioxygenase is indeed an intermediate of the reaction. By studying the reaction using stopped-flow techniques the spectrum of the short-lived intermediate was determined (10). Furthermore, when the overall reaction rate was compared to the amount of

complex formation at different time intervals, a linear plot was obtained, indicating that the oxygenated complex is definitely an intermediate of the reaction (10).

The following reaction sequence may account for all of the observations (11):



T: tryptophan

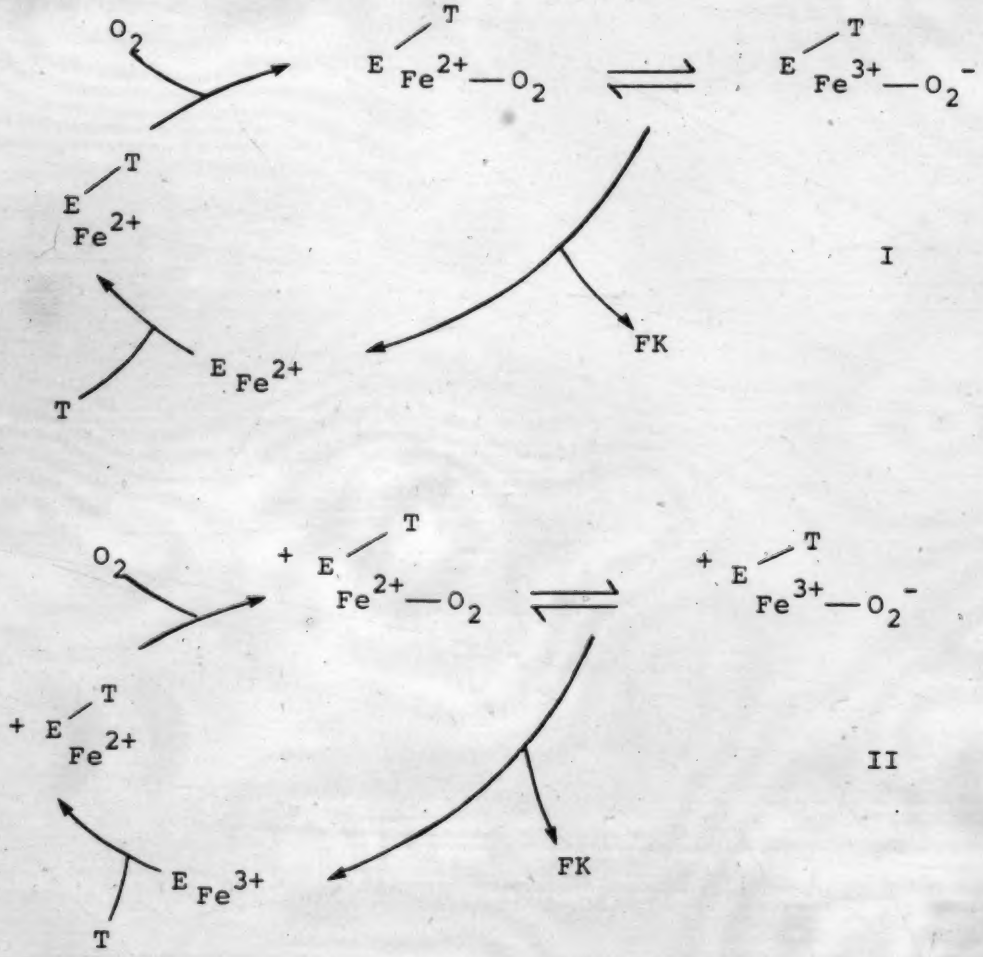
The organic substrate, tryptophan, first combines with the enzyme; the oxygen molecule is then bound to form a ternary complex of enzyme heme, tryptophan and oxygen. It is important to note, however, that the binding site of tryptophan is not clear. The fact that the appearance of an oxygenated intermediate is dependent on the presence of tryptophan implies that either tryptophan combines directly with the heme or elsewhere on the enzyme, evoking a conformational change and thereby increasing the reactivity of the heme moiety towards oxygen (7). This interpretation is supported by studies with cyanide and carbon monoxide where the presence of tryptophan in the system enhances the binding of these ligands to the enzyme (7).

A.3 Allosteric Behaviour of Tryptophan Dioxygenase

The activity of tryptophan dioxygenase was measured as a function of tryptophan concentration, yielding a sigmoidal curve which suggests the existence of and possible inter-

action between more than one tryptophan binding site on the enzyme (12). When α-methyltryptophan, an analogue of tryptophan, was added to the system, the rate of product formation was considerably increased at low tryptophan concentrations; whereas, it had no catalytic effect at very high tryptophan concentrations (12). With this addition of α-methyltryptophan, the sigmoidal shape of the curve disappeared and a classical hyperbolic tryptophan saturation curve resulted. Further experiments showed that α-methyltryptophan did not inhibit tryptophan dioxygenase activity (12). It was therefore concluded that α-methyltryptophan is neither an inhibitor nor a substrate of the enzyme and it presumably binds to a site on the enzyme, other than the catalytic site (12). Investigations also showed that the binding of tryptophan or α-methyltryptophan to the non-catalytic or allosteric site modifies the enzyme's interaction with oxygen (12).

The use of α-methyltryptophan to saturate the allosteric site enabled a study of carbon monoxide inhibition of oxygen (14). The results indicated uncompetitive inhibition by carbon monoxide with respect to tryptophan; an ordered bi-uni mechanism where tryptophan binds before oxygen is compatible with the findings (14). As shown in the following scheme two explanations for this bi-uni process were provided (14):



T: tryptophan FK: N-formylkynurenine

In scheme I the heme exists in a divalent state before and after tryptophan binds. In scheme II, the valence state of the heme may cycle during the reaction, with tryptophan responsible for the reduction of the heme iron. Only this second explanation is consistent with the fact that carbon monoxide inhibition with respect to tryptophan is uncompetitive. For scheme I there is no reason why carbon monoxide or oxygen could not bind to the ferrous heme (14). This

result is rather surprising since it had previously been shown that only the ferrous form of the enzyme is catalytically active (6).

A.4 Controversial Studies on the Heme Iron Valence State and Copper Presence in the Enzyme

A preliminary study of the valence state of tryptophan dioxygenase was carried out in 1959 (6). It was proposed that the native ferric enzyme is catalytically inactive and that ascorbate is required to reduce the native enzyme to its active ferrous form. By reacting the native ferric enzyme (Soret maximum at 403 nm) with tryptophan and ascorbic acid, a new spectrum with a Soret band at 433 nm was recorded. This new spectral species was associated with the ferrous form of the enzyme since reduction by H_2/Pd or sodium dithionite of the native enzyme yielded the same spectrum (6). Additional evidence was provided by studies on cyanide and carbon monoxide binding (6), catalase inhibition (6) and enzyme reconstitution (27). All of these early results pointed to one very important conclusion: only the enzyme in its divalent state is catalytically active.

A major dispute concerning the valence state of the enzyme during catalysis arose early in the history of tryptophan dioxygenase and was not resolved until recently. Two research groups, one in Japan and the other in the U.S.A., have collaborated and come to terms with the pro-

blem after many years of disagreement. However, for almost a decade the American laboratory strongly opposed the idea that only the ferrous form of the enzyme is catalytically active; it also claimed that copper was a cofactor of the enzyme (5,16,18-20,23,25,30).

The discovery by Feigelson and coworkers in the U.S.A. of two moles of copper per mole of enzyme and its apparent requirement for catalytic activity led them to predict that it was the oxidation state of the copper and not that of the heme iron which governed the enzyme's activity (5). They claimed to have isolated three redox forms of tryptophan dioxygenase (19,20). By treatment with an oxidizing agent, ferricyanide, the fully-oxidized form, $(Fe^{3+})_2(Cu^{2+})_2$ was produced. The catalytically active fully-reduced form, $(Fe^{2+})_2(Cu^{+})_2$, was easily obtained by the addition of sodium dithionite under anaerobic conditions. The half-reduced form, $(Fe^{3+})_2(Cu^{+})_2$, which also exhibited catalytic activity, was the most difficult to prepare. Having obtained the two catalytically active forms of the enzyme, the main concern was the determination of the roles of copper and heme components as substrate binding sites (19). By studying the competitive inhibition of oxygen by carbon monoxide, it was shown that both the fully-reduced and the half-reduced forms of the enzyme were inhibited to the same degree (19). It was hence postulated that for the half-reduced form of tryptophan dioxygenase, oxygen and its analogous substrates were bound to a site on the enzyme

other than the heme moieties. It was assumed that copper, the presence of which had previously been determined, was this second binding site. The proposed structures for the oxy and carboxy complexes of half-reduced and fully-reduced tryptophan dioxygenase were represented by the following scheme (20):

Half-reduced		Fully-reduced
Fe^{3+}	$O_2 - Cu^+$	$Fe^{2+} - O_2 - Cu^+$
Fe^{3+}	$CO - Cu^+$	$Fe^{2+} - CO - Cu^+$

It was believed that the oxygen (or carbon monoxide) acted as a bridging molecule between copper and heme. Depending on the oxidation state of the iron, the oxygen was more or less exclusively bound to the heme. If the heme were oxidized the oxygen molecule would lie in closer proximity to the cuprous ion.

Feigelson's discovery of copper in the enzyme was not, however, supported by the Japanese workers in the field, led by Hayaishi (17,21,22,24,31). Using similar techniques for copper analysis, only trace amounts of copper were found in Hayaishi's enzyme preparations (17,22,31). It was subsequently suggested that Feigelson's enzyme contained contaminant copper proteins with molecular properties similar to those of tryptophan dioxygenase (22).

In a very recent publication (32) the issue was finally settled. During a visit to the American laboratory, Hayaishi proved that his method of enzyme separation from

both rat liver and bacteria yields enzyme preparations which exhibit full catalytic activity but contain no copper. Likewise, Feigelson was successful in producing active, copper-poor enzyme samples, simply by the addition of ethylenediaminetetraacetate to the buffer solutions in order to remove contaminant metals. The agreement was established only through open communication and direct co-operation of the two laboratories involved (32).

A.5 Summary

In most recent years, little work, with the exception of the latest paper (32), has been carried out on the catalytic mechanism of tryptophan dioxygenase. Nevertheless, the stand-off between the Japanese and American groups has been put to rest and several definite conclusions may be drawn:

1. An oxygenated intermediate plays a major role in catalysis.
2. Tryptophan binds with the enzyme before oxygen.
3. The enzyme exhibits allosteric behaviour.
4. Copper is not an obligatory cofactor of the enzyme.

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